Investigation of $CD8^+$ T Cell Priming by Two Commonly Used Vaccinia Virus Strains

Yik Chun Wong

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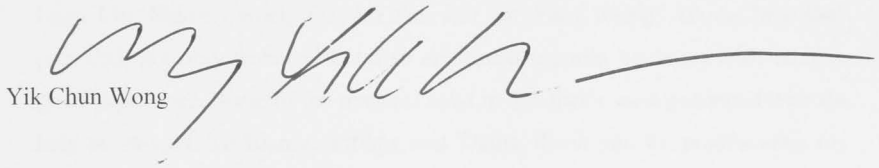
Declaration

This is to certify that,

- The work presented in thesis was conducted in the Australian National University in the laboratory of Associate Professor David C. Tscharke. This thesis comprises my original work towards the degree of Doctor of Philosophy except where indicated.

- To the best of my knowledge, the thesis does not contain material previously published by another person, except where due acknowledgment is made in the text.

- The thesis is less than 100,000 words in length, exclusive of figures, tables, references and appendices.



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Abstract

$CD8^+$ T cells help to control virus infections and tumours. Two main pathways, namely direct and cross presentation, are involved in generating and presenting antigenic peptides to induce $CD8^+$ T cell responses. In general, antigens that require minimal processing or are rapidly degraded allow better direct presentation while only stable antigens can be cross presented. Currently, several vaccinia virus (VACV) recombinants are being tested in clinical trials as vaccines to induce protective immunity, including $CD8^+$ T cell responses, against heterologous pathogens and cancers. The current paradigm, based on work with the virulent VACV strain Western Reserve (WR), suggests an important role of direct presentation for $CD8^+$ T cell priming. However, a study has reported that cross presentation is the major pathway for priming $CD8^+$ T cells against the highly attenuated modified VACV Ankara (MVA), a candidate vaccine vector. Understanding this disparity between WR and MVA may improve the design of VACV-based recombinant vaccines.

The priming pathway of native antigens expressed from WR and MVA was first examined in mice pre-treated with Toll-like receptor 9 ligand or cytochrome c. These methods have been reported to selectively inhibit cross presentation. However, results in this thesis demonstrate that these treatments also suppress direct presentation. Further investigations show that the extent of inhibition on $CD8^+$ T cell priming by CpG is dependent on the immunisation route, the viral replicative ability and, more crucially, the immunisation dose. Similarly, the replicative ability of VACV influences the inhibition effect of cytochrome c treatment. Overall, these two strategies do not reveal how VACV antigens are presented.

The immunogenicity of recombinant antigens expressed as minigenes from WR and MVA was then examined. The magnitude of $CD8^+$ T cell responses induced by the minigenes, which can only be directly presented, are at least similar to, if not higher than, the full-length antigens when expressed from WR and MVA. Contrary to the published work, the model antigen ovalbumin expressed as a minigene can be more immunogenic than the full-length antigen when expressed from MVA. This suggests that MVA behaves similarly to WR to prime $CD8^+$ T cells, i.e. by direct presentation.

We further demonstrate that the location where the transgene encoding the foreign antigen is inserted in the MVA genome determines its immunogenicity.

Two unexpected phenomena observed in this thesis were also explored. Firstly, a $CD8^+$ T cell response to an immunogenic peptide of influenza A virus, known as NP-366, can only be induced if the peptide is processed from a full-length protein from WR, but not as a minigene. This contradicts our studies described above and most of the VACV literature. Several hypotheses were tested to identify the possible mechanisms responsible. Secondly, we found that completely inactivated VACV induces $CD8^+$ T cell responses in vivo. Its application as a recombinant vaccine was examined.

In summary, this thesis has expanded our understanding on how antigens expressed from VACV strains WR and MVA are presented for $CD8^+$ T cell priming. The findings presented here provide important insights for VACV-based recombinant vaccine design.

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Abbreviations

#	Number
°C	Degree Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
β_2m	β_2-microglobulin
AIRE	Autoimmune regulator
AMT	4'-Aminomethyltrioxsalen hydrochloride
APC	Allophycocyanin
APCs	Antigen-presenting cells
Bim	B-cell lymphoma 2 interacting mediator of cell death
BMDC	Bone marrow-derived dendritic cell
bp	base pairs
bsd	Blasticidin resistant protein
BUdR	5-bromodeoxyuridine
CCR	CC-chemokine receptor
CFSE	5(6)-carboxyfluorescein diacetate N-succinimidyl ester
Clec9A	C-type lectin domain family 9 member A
CMC	Carboxymethyl cellulose
CpG	Synthetic oligonucleotide containing unmethylated cytosine-guanine dinucleotide motif
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CVA	Chorioallantois vaccinia virus Ankara
cytc	Cytochrome c
D	Diversity
D0	Dulbecco's modified Eagle medium without foetal bovine serum
D2	Dulbecco's modified Eagle medium supplemented with 2% (v/v) foetal bovine serum
D10	Dulbecco's modified Eagle medium supplemented with 10% (v/v) foetal bovine serum
DC	Dendritic cell
delIII	Deletion III
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
eGFP	Enhanced green fluorescent protein
eGFP-SKS	A recombinant antigen, consists of an enhanced green fluorescent

	protein, followed by the OVA-257, D3E-128 and gB-498 peptides
eGFP-STS	A recombinant antigen, consists of an enhanced green fluorescent protein, followed by, OVA-257, B8-20 and gB-498 peptides
Env	Envelope
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum aminopeptidase associated with antigen processing
ERAD	Endoplasmic reticulum-associated protein degradation
ERAP	Endoplasmic reticulum aminopeptidase
EDTA	Ethylenediaminetetraacetic acid
F5	A transgenic mouse strain expressing T cell receptors specific for the NT60NP-366 peptide presented on H-2D^{b}
FBS	foetal bovine serum
Fc	Fragment crystallizable region (Fc) of antibody
FSC	Forward scatter
g	Gram or Gravitational acceleration
Gag	Group-specific-antigen
gB	Glycoprotein B
GFP	Green fluorescent protein
h	Hour
HAU	hemagglutinin unit
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
hi	High
HIV	Human immunodeficiency virus
HSP	Heat shock protein
HSV	Herpes simplex virus
i.d.	Intradermal
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
IAV	Influenza A virus
IFN-I	Type I interferon
IFN-γ	Interferon-γ
IL	Interleukin
IRF4	Interferon regulatory factor 4
ITAM	Immunoglobulin receptor family tyrosine-based activation motif
J	Joining
KLRG1	Killer cell lectin-like receptor G1
L	Litre
LB	Luria-Bertani

Lck	Lymphocyte-specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
LM	*Listeria monocytogenes*
LNs	Lymph nodes
lo	Low
M	Molar
m.o.i.	Multiplicity of infection
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
mg	Milligram
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
min	Minute
ml	Millilitre
mM	Millimolar
MVA	Modified vaccinia virus Ankara
MyD	Myeloid differentiation marker
n	Number of samples
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
ng	Nanogram
NIAID	National Institute of Allergy and Infectious Diseases
NK	Natural killer
nm	Nanometre
nM	Nanomolar
NP	Nucleoprotein
NP-S-GFP	A recombinant antigen consisting of the PR8NP antigen, OVA-257 peptide and the green fluorescent protein
NS2	Non-structural-2
NT60	A/Northern Territory/60/1968 (a strain of influenza A virus)
NT60NP	Nucleoprotein from influenza A virus strain A/Northern Territory/60/1968
OT-I	A transgenic mouse strain expressing T cell receptors specific for the OVA-257peptide presented on H-2D^{b}
OVA	Ovalbumin
p	Probability value
PA	Acid polymerase
PB1	Basic polymerase 1
PB1F2	Basic polymerase 1 frame shift 2
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction

PD-1	Programmed cell death-1
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-cyanine 5
PE-Cy7	Phycoerythrin-cyanine 7
PFU	Plaque-forming unit
Pol	Polymerase
pp	Pages
PR8	A/Puerto Rico/8/1934 (a strain of influenza A virus)
PR8NP	Nucleoprotein from Influenza A virus strain A/Puerto Rico/8/1934
Puma	p53-up-regulated modulator of apoptosis
RAG	Recombination activating genes
RNA	Ribonucleic acid
sec	Second
SEM	Standard error of the mean
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SOC	Super optimal broth with catabolite repression
SSC	Side scatter
TAE	Tris-acetate-EDTA
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TK	Thymidine kinase
TLR	Toll-like receptor
TMP	4,5,8-Trimethylpsoralen
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Tyr	Tyrosinase
UbR-NP-S-GFP	The recombinant NP-S-GFP antigen tagged with an ubiquitin protein and an arginine residue at the amino-terminus.
V	Variable
VACV	Vaccinia virus
VSV	Vesicular stomatitis virus
v/v	Volume/volume
WR	Western Reserve
w/v	Weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1 Introduction

1.1 Innate and adaptive immune systems to fight infections

The mammalian immune system is a complex defence mechanism that eliminates tumour cells and fights infections caused by a wide range of pathogens including viruses. The immune system is comprised of innate immunity and adaptive immunity. Both are essential for controlling infections.

Innate immunity is the first defence system employed during a pathogenic infection. Although it was thought to function non-specifically, it still needs to recognise incoming pathogens in order to target them (Akira et al., 2006). Certain components available across different pathogens, including bacterial and viral DNA, RNA, lipopolysaccharides and proteins, can be recognised by the innate immune system. These are called pathogen-associated molecular patterns (Medzhitov and Janeway, 1997). Once the innate immune system is stimulated, multiple arms of the innate immune system will start to mediate pathogen clearance. These include (i) antimicrobial peptides to limit the infectivity of pathogens and stop their replication (Klotman and Chang, 2006), (ii) complement components to opsonise and lyse bacteria and viruses (Blue et al., 2004), (iii) the secretion of cytokines by multiple cell types which have direct anti-pathogenic properties (Guidotti and Chisari, 2000) , and (iv) a range of innate immune cells, including neutrophils and macrophages, that phagocytose virions and kill infected cells (Guidotti and Chisari, 2001).

During the early infection period when infection control is largely the duty of the innate immune system, the adaptive immune system is activated and becomes functional. The heart of this arm of immunity is the ability of a subset of immune cells to recognise a particular antigen via their unique antigen-binding receptors and the selective expansion of these cells to specifically target the invading pathogen (Burnet, 1957). This theory is known as clonal selection and has been confirmed by years of research. After clearance of an infection, memory is provided to allow rapid secondary responses against the same antigens if encountered later. Adaptive immunity can be subdivided into two branches: (i) humoral immunity, which involves antibodies produced by B cells that develop in the bone marrow (Mitchell and Miller, 1968; Nossal et al., 1968; Roitt et al.,

1969; Playfair and Purves, 1971; Ryser and Vassalli, 1974), and (ii) cell-mediated immunity, which involves the functions exerted by T cells that develop in the thymus (Roitt et al., 1969; Bevan, 1977; Zinkernagel et al., 1978a). Both arms of adaptive immunity are essential for controlling virus infection (Guidotti and Chisari, 2001).

The innate and adaptive immune systems do not act independently. They engage in crosstalk to regulate the overall immune response induced during an infection. For instance, direct recognition of pathogen-associated molecular patterns by a specialised cell type, called dendritic cells (DCs), leads to their activation, enabling them to induce potent cell-mediated adaptive immunity (Sporri and Reis e Sousa, 2005; Kratky et al., 2011). In addition, neutrophils have been found to facilitate T cell responses via activation of DCs (van Gisbergen et al., 2005). Reciprocally, the adaptive immune response can also enhance innate immunity. One example is that the activity of complements can be induced by antibodies complexed with antigens (Porter and Reid, 1978; Ziccardi, 1984; Duncan and Winter, 1988). As another example, activated T cells can trigger the recruitment and activation of macrophages (Stout and Bottomly, 1989; Cantor and Haskins, 2007; Nishimura et al., 2009). Collectively, all arms of the immune system function as a whole to protect the host against disease.

1.2 T cells in the cell-mediated adaptive immunity

There are two main types of T cells involved in the cell-mediated adaptive immunity and they are distinguished based on the co-receptors they express on the cell surface: $CD4^+$ and $CD8^+$ T cells. Notably, these T cells play different roles in the immune response to infection.

1.2.1 $CD4^+$ T cells

$CD4^+$ T cells are sometimes referred to as helper T cells because of their potential involvement in promoting other branches of the immune response, such as antibody production by B cells (Tony and Parker, 1985; Hodgkin et al., 1990) and $CD8^+$ T cell immunity (Husmann and Bevan, 1988). $CD4^+$ T cells recognise

peptides presented on major histocompatibility complex class II molecules (MHC-II) on the surface of antigen-presenting cells (APCs) through their T cell receptors (TCRs; Ziegler and Unanue, 1981; Allen et al., 1984; Babbitt et al., 1985; Reinherz et al., 1999). These cells can be further subdivided into several subsets based on the cytokines they produce and their main immune functions, namely type 1, type 2, interleukin(IL)-17-producing $CD4^+$ T cells, and follicular helper T cells (Mosmann et al., 1986; Breitfeld et al., 2000; Schaerli et al., 2000; Harrington et al., 2005; Park et al., 2005). Besides promoting immunity, some $CD4^+$ T cells, namely regulatory T cells, can suppress the magnitude of immunity and play a role in establishing tolerance (Sakaguchi et al., 1995; Thornton and Shevach, 1998).

1.2.2 $CD8^+$ T cells

$CD8^+$ T cells are directly involved in controlling infection by intracellular pathogens. Activated effector $CD8^+$ T cells can lyse infected cells via perforin and Fas pathways (Kagi et al., 1994; Lowin et al., 1994). They also secrete cytokines which have direct anti-microbial properties (Morris et al., 1982; Guidotti et al., 1996; Badovinac et al., 2000; Slifka and Whitton, 2000a). Furthermore, various chemokines can be expressed by activated $CD8^+$ T cells, which in turn help attract other immune cells to the site of infection (Cocchi et al., 1995; Conlon et al., 1995; Wagner et al., 1998; Cook et al., 1999). In addition to clearing infections, $CD8^+$ T cells are also involved in fighting tumours (Anichini et al., 1987). In order to exert these functions, $CD8^+$ T cells must first recognise peptides presented on major histocompatibility complex class I molecules (MHC-I) on the surface of APCs (Zinkernagel and Doherty, 1974; Townsend et al., 1986b).

1.3 Interaction between a $CD8^+$ T cell and its ligand

1.3.1 Peptide/MHC-I complex: the ligand recognised by $CD8^+$ T cells

MHC-I is a heterodimer involved in presenting peptides on the surface of a cell. It consists of a membrane-anchored heavy chain and a soluble β_2-microglobulin (β_2m) that are non-convalently linked together (Cresswell et al., 1973; Grey et al., 1973; Peterson et al., 1974; Silver and Hood, 1974). There are three extracellular

domains in the heavy chain: the α_1 and α_2 domains form the top of the molecule where an antigenic peptide can bind, while the α_3 domain extends from the membrane anchoring sequence and associates with β_2m (Bjorkman et al., 1987b). The α_3 domain is also the site that the CD8 co-receptor molecule on T cells binds (Connolly et al., 1988; Potter et al., 1989; Connolly et al., 1990; Salter et al., 1990). The MHC-I heavy chain is polygenic and highly polymorphic, allowing each individual host to express several variants of MHC-I (Lawlor et al., 1990). These two features of MHC-I ensure that a diverse set of peptides can be presented within individual hosts and across the whole population.

In term of the three-dimensional structure, the α_1 and α_2 domains of MHC-I are folded into a tertiary structure containing two parallel α-helices, sitting on top of a β-pleated sheet, forming a groove for peptide binding (Bjorkman et al., 1987a; Bjorkman et al., 1987b). Within this groove, there are several regions, known as "pockets", which interact with particular amino acids along the bound peptide (Fremont et al., 1992; Matsumura et al., 1992; Zhang et al., 1992). To secure the binding of a peptide, multiple hydrogen bonds are formed between both termini of the peptide and the binding pockets at the two ends of the groove (Madden et al., 1993). Amino acid residues found in the pockets along the groove of MHC-I are polymorphic and they allow allele-specific interactions with anchor residues in the peptides to determine the sequence specificity of MHC-I (Matsumura et al., 1992; Madden et al., 1993). The presented peptides that can be recognised by $CD8^+$ T cells during an immune response are termed '$CD8^+$ T cell epitopes'.

1.3.2 T cell receptors recognise peptide/MHC-I complexes

$CD8^+$ T cells express TCRs that recognise peptides presented on MHC-I molecules on the surface of APCs (Bjorkman et al., 1987b). Each TCR is a heterodimer consisting of a TCR-α and a TCR-β chain linked by a disulfide bond (Allison et al., 1982; Haskins et al., 1983; Meuer et al., 1983; Chien et al., 1984; Hedrick et al., 1984; Saito et al., 1984; Yanagi et al., 1984). Each TCR chain is folded into variable and constant domains, and the top of the variable domain interacts directly with the exposed amino acid residues of the MHC-I-bound peptides and some regions of the α-helices of MHC-I (Garboczi et al., 1996;

Garcia et al., 1996; Ding et al., 1998; Rudolph et al., 2006). The CD8 co-receptor also binds to MHC-I to increase stability of the TCR-peptide/MHC-I interaction (Luescher et al., 1995; Daniels and Jameson, 2000). T cells can also express TCR consisting of TCR-γ and δ chains and these cells represent a separate lineage from the TCR-α/β-expressing T cells, with specialised functions (Lew et al., 1986; Born et al., 1987; Janis et al., 1989). Conventional TCR-α/β T cells will be the focus here.

In order to recognise all the possible peptide/MHC-I complexes, a diverse repertoire of T cells, each expressing a TCR with a unique specificity, is needed. This diversity is generated by the following mechanisms. Firstly, there are multiple variable (V), diversity (D; only for β chain), and joining (J) gene segments in each TCR chain locus (Toyonaga et al., 1985; Yoshikai et al., 1985). The variable domain of a TCR chain is formed by random rearrangement and recombination of each of these gene segments (Chien et al., 1984; Hedrick et al., 1984; Saito et al., 1984; Yanagi et al., 1985). This variable domain is joined with a constant domain, which contains a transmembrane region for anchoring to the cell membrane (Malissen et al., 1984). This recombination process was firstly identified for the generation of the antibody-encoding gene in B cells, and similar to TCRs, this process allows the generation of antibodies with a diverse specificity (Hozumi and Tonegawa, 1976; Brack et al., 1978). V(D)J rearrangement is initiated by two related enzymes called recombination activating genes (RAG) 1 and 2, which allow the cleavage of the double-stranded deoxyribonucleic acid (DNA) around the gene segments (Schatz et al., 1989; Oettinger et al., 1990). The ends of the DNA are then joined by several DNA ligases (Taccioli et al., 1993; Baumann and West, 1998). This recombination process allows gene segments that are far apart to be joined to form the final TCR genes by removing the DNA sequences in between DNA breaks (Chien et al., 1984; Hedrick et al., 1984; Saito et al., 1984). Secondly, nucleotides can be inserted into or removed from the junctions between gene segments to increase the diversity of TCR genes (Lieber et al., 1988; Lafaille et al., 1989; Feeney, 1991). Part of the process is catalysed by terminal deoxynucleotidyl transferase, which adds random nucleotides to the ends of DNA breaks (Desiderio et al., 1984; Kallenbach et al., 1992; Gilfillan et al., 1993; Komori et al., 1993).

When a productive TCR chain is generated from a rearranged gene on one chromosome, the rearrangement of this allele on the other chromosome will be stopped (Uematsu et al., 1988). This allelic exclusion of the TCR ensures that each T cell only expresses one form of TCR-α/β molecule with a single specificity in most cases. However, this process is not absolute as T cells may express two functional TCR-α or β chains in some cases (Padovan et al., 1993; Balomenos et al., 1995; Elliott and Altmann, 1995; Heath et al., 1995). After generating a diverse range of TCRs by V(D)J recombination, a selection process is then required in the thymus to eliminate unwanted rearranged products and keep those that can recognise self MHC-I complexed with peptides.

1.4 Development of $CD8^+$ T cells in thymus

To create a diverse set of $CD8^+$ T cells that can recognise foreign, but not self, peptides presented on MHC-I, T cells are subjected to two selection processes during their development in the thymus. They are called positive and negative selection. Firstly, T cell progenitors migrate from the bone marrow to the thymus via the blood stream (Moore and Owen, 1967; Doria and Agarossi, 1969; Le Douarin and Jotereau, 1975). These newly-arrived progenitors do not express CD4 or CD8 co-receptors and are yet to express TCRs (Bluestone et al., 1987). These cells undergo TCR-β chain rearrangement followed by TCR-α chain rearrangement (Petrie et al., 1995). The TCR-α chain locus can undergo multiple rounds of V(D)J recombination, allowing for selection of a functional TCR that can successfully recognise peptides presented on self MHC-I in the thymus when paired with a TCR-β chain (Borgulya et al., 1992; Brändle et al., 1992; Petrie et al., 1993).

T cells expressing TCR-α/β molecules are first subjected to positive selection and only those carrying TCRs that bind to self peptide/MHC-I molecules with sufficient affinity will survive, forming a pool of $CD8^+$ T cells that are restricted to self MHC-I (Bevan, 1977; Fink and Bevan, 1978; Zinkernagel et al., 1978b). T cells at this stage express both CD4 and CD8 co-receptors and they are positively selected against the cortical thymic epithelial cells which express self

peptide/MHC-I as the ligands for this process (Fink and Bevan, 1978; Zinkernagel et al., 1978b; Lo and Sprent, 1986; Anderson et al., 1994; Wilkinson et al., 1995). T cells expressing TCRs that fail to bind to self peptide/MHC-I will die by neglect via apoptosis while T cells with functional TCRs will express the anti-apoptotic Bcl-2 protein and survive (Kisielow and Miazek, 1995). During this process, the CD4/CD8-lineage fate of the T cells is also determined, such that T cells recognising peptide/MHC-I will express the CD8 co-receptor only and become $CD8^+$ T cells (Teh et al., 1988; reviewed Singer et al., 2008). The surviving $CD8^+$ T cells then undergo negative selection in which those recognising self peptide/MHC-I with too high affinity are eliminated (Ashton-Rickardt et al., 1994; Sebzda et al., 1994). This process happens in the cortex as well as the medulla region of the thymus and involves DCs and thymic epithelial cells as the APCs (Gao et al., 1990a; Webb and Sprent, 1990; Gallegos and Bevan, 2004; Mayerova and Hogquist, 2004; McCaughtry et al., 2008; Le Borgne et al., 2009; Hubert et al., 2011). Autoreactive T cells are removed by this process to induce central tolerance and prevent autoimmunity. To induce tolerance against tissue-specific antigens, medullary thymic epithelial cells express a protein called autoimmune regulator (AIRE). AIRE allows expression of antigens normally produced in peripheral tissues only (Anderson et al., 2002; Liston et al., 2003).This allows for negative selection against tissue-specific autoreactive $CD8^+$ T cells (Gallegos and Bevan, 2004; Hubert et al., 2011). T cells that are selected against during negative selection are eliminated by apoptosis (Surh and Sprent, 1994). This process involves expression of several pro-apoptotic molecules, including B-cell lymphoma 2 interacting mediator of cell death (Bim) and p53-up-regulated modulator of apoptosis (Puma; Bouillet et al., 1999; Bouillet et al., 2002; Erlacher et al., 2006; Gray et al., 2012). Overall, the surviving $CD8^+$ T cells express TCRs that weakly recognise self peptide/MHC-I, ensuring that they can recognise self MHC-I, but not react to self antigens.

1.5 Activation of naive $CD8^+$ T cells in periphery

Once $CD8^+$ T cells are developed, they are released into the circulatory system. It is generally accepted that these cells are fully mature based on the similar

phenotypes and potential functionality between T cells that have recently left the thymus and peripheral T cells isolated from secondary lymphoid organs (Scollay et al., 1978; Scollay, 1982; Scollay et al., 1984). However, more recent studies suggest that some T cells continue the maturation process within secondary lymphoid organs (Boursalian et al., 2004; Houston et al., 2008). In either scenario, naive mature T cells are available in the periphery. To survive in the periphery and maintain their homeostatic proliferation ability, naive $CD8^+$ T cells require TCR signalling by interacting weakly with self peptide/MHC-I on peripheral cells (Tanchot et al., 1997; Goldrath and Bevan, 1999; Kieper and Jameson, 1999; Murali-Krishna et al., 1999). The cytokine IL-7 is also required for T cell homeostatic proliferation and survival (Schluns et al., 2000; Tan et al., 2001). These naive $CD8^+$ T cells circulate around the host and wait for activation in case of an infection.

During an infection, $CD8^+$ T cell activation mostly happens in secondary lymphoid organs, such as the spleen and lymph nodes (LNs). Naive $CD8^+$ T cells express various LN-homing receptors including CD62L and CC-chemokine receptor 7 (CCR7; Gallatin et al., 1983; Mobley and Dailey, 1992; Förster et al., 1999; Sallusto et al., 1999). As a result, naive $CD8^+$ T cells readily migrate between LNs via the blood and lymph (Mackay et al., 1990; Weninger et al., 2001). In addition, secondary lymphoid organs are architecturally organised to allow optimal opportunities for $CD8^+$ T cells to interact with specialised APCs, mainly DCs, for $CD8^+$ T cell activation (Schaefer et al., 2001; Bousso and Robey, 2003; Mempel et al., 2004). Induction of $CD8^+$ T cell responses against viral and bacterial infections is significantly impaired in splenectomised mice and lymphotoxin α-deficient mice, which lack proper secondary lymphoid organs (Karrer et al., 1997; Suresh et al., 2002; Klonowski et al., 2006), demonstrating the importance of these organs for $CD8^+$ T cell activation.

While MHC-I is expressed on many cell types, but it is generally accepted that naive $CD8^+$ T cells can only be activated (or primed) after recognising cognate peptides presented on MHC-I by professional APCs. Although macrophages have been considered as professional APCs for activating $CD8^+$ T cells, targeted depletion of DCs inhibits $CD8^+$ T cell immunity during various types of infections

(Jung et al., 2002; Probst and van den Broek, 2005; Ciavarra et al., 2006; Kassim et al., 2006; John et al., 2009; Hickman et al., 2011b). In addition, clustering of $CD8^+$ T cells around DCs that leads to $CD8^+$ T cell activation has been observed as early as six hours after infection of mice with vaccinia virus (VACV) and vesicular stomatitis virus (VSV; Norbury et al., 2002; Hickman et al., 2008). Although naive $CD8^+$ T cells can interact stably with infected macrophages in draining LNs after VACV infection if DCs are depleted, this interaction does not allow full activation and effector differentiation of the $CD8^+$ T cells (Hickman et al., 2011b). These data suggest an essential role for DCs in priming $CD8^+$ T cell immunity in vivo. In support of this model, DCs have several characteristics that are essential for activating T cells, including the expression of co-stimulatory molecules and the necessary machinery for presenting antigens. These features are discussed in Sections 1.5.2 and 1.7 respectively.

To prime a productive $CD8^+$ T cell response, three separate yet integrated signals from DCs are required, namely signalling from the TCR-peptide/MHC-I complex interaction, signal 2 from co-stimulatory molecules, and signal 3 from inflammatory cytokines.

1.5.1 Signal 1: TCR signalling pathway

Once a TCR on a naive $CD8^+$ T cell recognises a peptide/MHC-I complex on an APC, several signalling cascades will be activated to induce $CD8^+$ T cell activation. Many of the studies on the TCR signalling pathway have focused on a $CD4^+$ T cell leukaemia cell line known as Jurkat cells (Abraham and Weiss, 2004). However, the TCR signalling transduction pathway is conserved between $CD4^+$ and $CD8^+$ T cells. On the T cell membrane, the TCR-α/β dimer forms a larger complex with multiple CD3 subunits, including the CD3γ, δ, ε, ζ chains (Borst et al., 1982; Kanellopoulos et al., 1983; Borst et al., 1984; Weiss and Stobo, 1984; Weissman et al., 1986). Each CD3 subunit has a cytoplasmic tail containing at least one immunoglobulin receptor family tyrosine-based activation motif (ITAM) which is important for TCR signalling (Reth, 1989; Romeo et al., 1992). Specific tyrosine residues in these motifs are phosphorylated during T cell activation, mainly by the lymphocyte-specific protein tyrosine kinase (Lck; Stein

et al., 1992; Straus and Weiss, 1992; Iwashima et al., 1994; van Oers et al., 1996a). Once the ITAMs of the CD3 subunits are phosphorylated, multiple adaptor molecules are recruited (Iwashima et al., 1994; Finco et al., 1998; Zhang et al., 1998a). Further signal transduction occurs via phosphorylation of these adaptors, resulting in increased expression of several important transcriptional factors required for T cell activation, including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), nuclear factor of activated T-cells and activator protein 1 (Jamieson et al., 1991; Chan et al., 1992; Iwashima et al., 1994; Wu et al., 1996; Finco et al., 1998; Zhang et al., 1998a).

To demonstrate the importance of this signalling pathway, T cell development in the thymus is arrested in mice lacking Lck or other adaptor molecules (Negishi et al., 1995; van Oers et al., 1996b; Zhang et al., 1999). To dissect the role of Lck in T cell activation after their development, a transgenic mouse model, in which the expression of Lck can be induced by doxycycline, has been used (Tewari et al., 2006). This study demonstrated that Lck expression is necessary for $CD8^+$ T cell activation for primary responses against lymphocytic choriomeningitis virus (LCMV) and VACV infections in vivo (Tewari et al., 2006). Surprisingly, the same study showed that the secondary anti-LCMV response seems to be independent of Lck expression although the authors noted that this could be influenced by the TCR affinity for its peptide/MHC-I ligand (Tewari et al., 2006).

The role of the duration of TCR signalling in determining $CD8^+$ T cell immunity has also been examined. It has been suggested that the TCR signalling pathway may simply be required for programming $CD8^+$ T cells into a stage that can undergo proliferation without the presence of further TCR stimulation (Mercado et al., 2000; Kaech and Ahmed, 2001; van Stipdonk et al., 2001; Wong and Pamer, 2001). On the other hand, during an acute infection, an antigen will be present for a period of time in the host before the pathogen is eliminated. Using an in vivo immunisation model involving transfer of peptide-pulsed DCs that could be depleted at various times, Prlic et al (2006) showed that the size of $CD8^+$ T cell immunity is directly proportional to duration of antigen presentation by MHC-I on DCs. Similarly, the magnitude of immunity is positively influenced by the dose of pathogen during infections and the duration of antigen expression in DNA

immunisation (Kaech and Ahmed, 2001; Badovinac et al., 2002; Hovav et al., 2007), suggesting that the length of TCR signalling is important for programming and tuning $CD8^+$ T cell immunity.

1.5.2 Signal 2: co-stimulatory signalling

Without appropriate co-stimulatory signals, T cells fail to proliferate even with the presence of TCR signalling and these T cells can be driven into an anergic state where they become non-responsive, even after subsequent antigen encounter (Weiss et al., 1984; Jenkins and Schwartz, 1987; Otten and Germain, 1991; Harding et al., 1992; Gett et al., 2003). Multiple co-stimulatory molecules have been identified, but the CD28-CD80/CD86 costimulatory pathway is the best studied. CD28 is expressed on $CD8^+$ T cells (Turka et al., 1990; Tan et al., 1992). When $CD8^+$ T cells are activated in vitro via TCR stimulation, engagement of CD28 with a stimulatory antibody increases the production of multiple cytokines by the T cells, including IL-2 which acts as an autocrine growth factor to promote T cell proliferation (Thompson et al., 1989). It was later found that CD80 and CD86 are the natural ligands for CD28 and they are expressed on DCs (Larsen et al., 1992; Hathcock et al., 1993; Caux et al., 1994b). The expression of these two ligands can be further enhanced after DC maturation (Larsen et al., 1992; Caux et al., 1994b; Larsen et al., 1994). By binding to CD28, these two ligands provide similar co-stimulation to T cells (Freeman et al., 1995; Lanier et al., 1995). Mechanistically, the CD28-CD80/CD86 interaction results in an activation of the pathways that are also involved in the TCR signalling transduction (Verweij et al., 1991; Su et al., 1994; Coudronniere et al., 2000; Michel et al., 2000). As a result, this co-stimulatory pathway helps to reduce the activation threshold required for inducing a productive TCR signal (Pardigon et al., 1998; Acuto and Michel, 2003).

CD80 and CD86 can also bind to another receptor called cytotoxic T lymphocyte antigen 4 (CTLA-4) which is highly expressed on the cell surface of activated T cells (Linsley et al., 1991; Linsley et al., 1992). The interaction of CD80/CD86 with CTLA-4 down-regulates co-stimulatory signal transduction and inhibits T cell proliferation and functions (Walunas et al., 1994; Walunas et al., 1996). It has

been recently shown that CTLA-4 on $CD4^+$ T cells can reduce surface expression of CD80 and CD86 on APCs by trans-endocytosing them into the T cells (Qureshi et al., 2011). This inhibitory receptor provides a balance of co-stimulation during T cell activation.

During infection by a pathogen, CD28-CD80/CD86 costimulation plays an important role in eliciting $CD8^+$ T cell immunity. $CD8^+$ T cell responses are altered, reduced and/or delayed if this costimulatory signal is missing during (a) viral infections, including ectromelia virus, murine γ-herpesvirus 68, herpes simplex virus 1 (HSV-1), influenza A virus (IAV), LCMV, VACV and VSV infection (Kündig et al., 1996; Edelmann and Wilson, 2001; Bertram et al., 2002; Fang and Sigal, 2006; Fuse et al., 2008; Salek-Ardakani et al., 2009; Grujic et al., 2010; Dolfi et al., 2011; Eberlein et al., 2012), (b) bacterial infection with *Listeria monocytogenes* (LM; Mittrücker et al., 2001; Shedlock et al., 2003), and (c) parasitic infections with *Toxoplasma gondii* and *Plasmodium chabaudi*, a cause of murine malaria (Villegas et al., 1999; Elias et al., 2005). In some early studies based on in vitro cytotoxicity alone, it was suggested that CD28 is not important for inducing primary $CD8^+$ T cell responses against LCMV and VACV (Shahinian et al., 1993; Kündig et al., 1996). However, it was later identified that responses against these viral infections are actually reduced and altered when antigen-specific cell numbers and their functionality were directly measured ex vivo using peptide/MHC-I tetramers and specific phenotypic markers (Fuse et al., 2008; Grujic et al., 2010).

Other co-stimulatory signalling molecules can also be involved in activating $CD8^+$ T cells, including interactions between CD137 and CD137 ligand, between CD27 and CD70, between OX40 and OX40 ligand, as well as between ICOS and B7h (reviewed in Watts, 2004; Chen and Flies, 2013). These co-stimulatory interactions play a role in priming optimal $CD8^+$ T cell responses. It should be noted that co-stimulatory interactions can be enhanced by $CD4^+$ T cells and this will be discussed in Section 1.6.1. Overall, the co-stimulatory pathways provide an essential companion signal for the TCR-peptide/MHC-I signal to induce $CD8^+$ T cell immunity.

1.5.3 Signal 3: Inflammatory signalling – the danger signal

The role of inflammatory signals in T cell activation is a relatively new discovery compared to the other two signals, and the mechanisms involved are still under extensive investigation. In vitro experiments demonstrated that an external supply of the inflammatory cytokine IL-12 can significantly enhance the in vitro proliferation, survival and effector functions of naive $CD8^+$ T cells when they are stimulated in the presence of the TCR and co-stimulatory signals (Curtsinger et al., 1999; Curtsinger et al., 2003a). This cytokine can replace conventional adjuvants to induce $CD8^+$ T cell immunity during in vivo peptide immunisation, which would otherwise induce a tolerant $CD8^+$ T cell state if there is no adjuvant (Curtsinger et al., 1999; Curtsinger et al., 2003b). Other than IL-12, type I interferon (IFN-I, including IFN-α and β) can also provide signal 3 to naive T cells (Curtsinger et al., 2005; Le Bon et al., 2006). Other types of signal 3, including IL-21 and interferon-γ (IFN-γ), have been proposed and their importance has started to emerge via recent studies (Whitmire et al., 2005; Sercan et al., 2006; Casey and Mescher, 2007). As these cytokines are produced by DCs after maturation (Cella et al., 1996), $CD8^+$ T cells will receive all the required signals by interacting with activated antigen-presenting DCs.

Signal 3 is also important to elicit optimal $CD8^+$ T cell immunity during pathogenic infections. Mutant TCR-transgenic $CD8^+$ T cells specific to a LCMV $CD8^+$ T cell epitope that lacked the IFN-I receptor showed a significant reduction in survival during the expansion phase compared with wildtype cells after adoptive transfer into mice that were subsequently infected with LCMV (Kolumam et al., 2005). A similar requirement for IFN-I has been reported for VACV, VSV, and LM infections, although the reduction in $CD8^+$ T cell expansion was not as dramatic as for LCMV (Thompson et al., 2006). Two reports have suggested that the dependence on IFN-I and IL-12 for $CD8^+$ T cell expansion is minimal during the primary response induced by VACV infection (Aichele et al., 2006; Keppler et al., 2012). In contrast, IL-12, but not IFN-I, is necessary for optimal memory and secondary $CD8^+$ T cell immunity to VACV infection (Xiao et al., 2009). These data imply that the type of pathogen influences the dependence of $CD8^+$ T cell immunity on the source of signal 3.

Collectively, all the three signals discussed above are required for the optimal priming of a $CD8^+$ T cell response. Loss of these signals severely affects the magnitude of response induced.

1.6 Progression of a $CD8^+$ T cell response during an acute infection

After $CD8^+$ T cells receive all the required signals for activation during an infection, they will differentiate and expand into effector cells to control infection. After the resolution of the infection, the effector $CD8^+$ T cell population goes through a contraction phase where only memory cells survive. The memory population provides protective immunity against future infections by the same pathogen. This phenomenon was first reported with experiments based on in vitro cytotoxicity assays (Marker and Volkert, 1973; Moskophidis et al., 1987; Oehen et al., 1992; Moskophidis et al., 1993; Lau et al., 1994; Tripp et al., 1995), and later confirmed by the direct measurement of antigen-specific $CD8^+$ T cells with peptide/MHC-I tetramers or ex vivo peptide stimulation followed by labelling of intracellular cytokines (Butz and Bevan, 1998; Murali-Krishna et al., 1998; Flynn et al., 1999; Harrington et al., 2002; Yang et al., 2003). The progress of a $CD8^+$ T cell response during an acute infection is illustrated in Figure 1-1.

A single naive CD8 T cell has the potential to differentiate into effector and memory cells, as demonstrated using a DNA-barcoding technique to label individual cells, as well as single cell transfer (Stemberger et al., 2007; Gerlach et al., 2010). This indicates that the fate of T cells is not imprinted during their development in the thymus, but is influenced by T cell activation. After interacting with APCs, naive $CD8^+$ T cells become activated and undergo asymmetric cell division, where the two daughter cells receive different amounts of molecules critical for the TCR signalling pathway and differentiation (Chang et al., 2007). This leads to the generation of cells that are committed to the effector and memory lineages from the same progenitors (Chang et al., 2007; Oliaro et al., 2010). Various pathways are involved in the expansion and development of naive $CD8^+$ T cells into effector cells and they are discussed below.

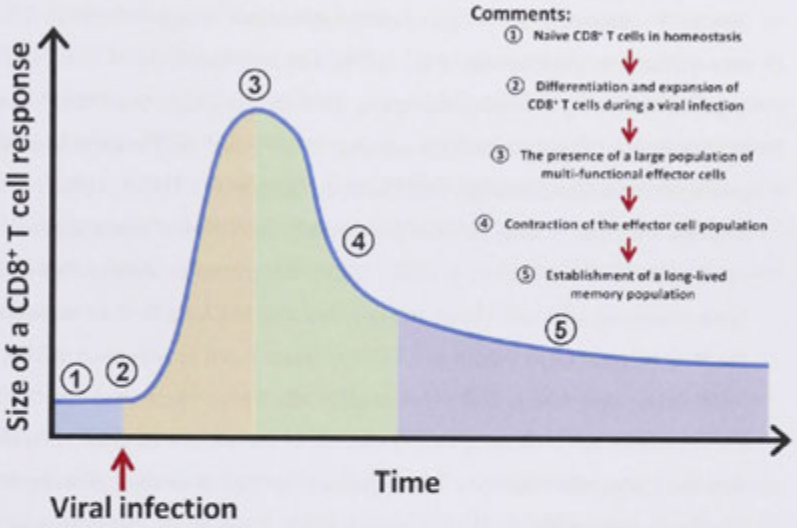


Figure 1-1| A CD8⁺ T cell response induced during an acute virus infection. (1) Within a host, there are naive CD8⁺ T cells that are maintained in homeostasis. (2) During an acute virus infection, some naive CD8⁺ T cells become activated via recognition of cognate peptides presented on professional APCs. These CD8⁺ T cells undergo proliferation and differentiation into effectors cells. (3) During the peak of the response, a large population of effector CD8⁺ T cells is available for controlling the infection. (4) After the clearance of the infection, the effector CD8⁺ T cell population contracts and only long-lived memory cells survive. (5) The established memory population is in a greater number than the naive population, allowing a rapid induction of response against subsequent infections with the same virus.

1.6.1 Factors involved in the expansion of antigen-specific CD8$^+$ T cells

When naive CD8$^+$ T cells are activated within LNs, expression of the early activation marker CD69 is induced on the cell surface (Yokoyama et al., 1988; Testi et al., 1989). CD69 can in turn down-regulate surface expression of sphingosine-1-phosphate receptor 1, a chemotactic receptor which is responsible for lymphocyte migration out from secondary lymphoid organs (Shiow et al., 2006). The reduced level of sphingosine-1-phosphate receptor 1 allows T cells to stay within LNs (Matloubian et al., 2004). The activated CD8$^+$ T cells will begin to proliferate in the LNs after interacting with antigen-presenting DCs (Mempel et al., 2004). IL-2 plays a role in promoting CD8$^+$ T cell proliferation here. IL-2 is produced as an autocrine growth factor by activated CD8$^+$ T cells, or can be supplied by CD4$^+$ T cells or DCs during early activation (Granucci et al., 2001; van Stipdonk et al., 2003; Lai et al., 2009). Early studies showed that IL-2 can drive expansion of T cell clones in vitro, and that blocking IL-2 or the α chain of the IL-2 receptor (also known as CD25) reduces T cell proliferation (Gillis et al., 1978; Mier and Gallo, 1980; Leonard et al., 1982; Stern and Smith, 1986). Expression of the IL-2 receptor is induced on the surface of CD8$^+$ T cells once they have been stimulated via TCR signalling in vitro or in vivo, allowing them to proliferate in response to IL-2 (Larsson, 1981; Robb et al., 1981; Hemler et al., 1984; D'Souza and Lefrançois, 2003; van Stipdonk et al., 2003; Kalia et al., 2010). During VACV infection in mice, IL-2 signalling via the IL-2 receptor is essential for the continuous proliferation and maximal expansion of primed CD8$^+$ T cells (Obar et al., 2010). Endogenous IL-2 expression by CD8$^+$ T cells themselves is important in this infection model, suggesting again a role for IL-2 as an autocrine factor (Feau et al., 2011). A similar requirement for IL-2 receptor signalling for optimal expansion of CD8$^+$ T cells is detected during LCMV and VSV infections, as well as immunisation with soluble antigens (D'Souza and Lefrançois, 2003; Bachmann et al., 2007; Pipkin et al., 2010). Moreover, further studies found that IL-2 is essential to induce protective secondary CD8$^+$ T cell immunity against LCMV and LM infections (Williams et al., 2006; Feau et al., 2011).

In addition to IL-2, it is generally accepted that CD4$^+$ T cells can facilitate CD8$^+$ T cell immunity. CD4$^+$ T cells express the CD40 ligand which can be further up-

regulated after activation (Roy et al., 1993; Lesley et al., 2006). This ligand is recognised by CD40 expressed on DCs and this will result in activation of DCs, including induction of co-stimulatory molecules and IL-12 expression (Caux et al., 1994a; Cella et al., 1996; Koch et al., 1996; Schulz et al., 2000). Antigen-presenting DCs activated via CD40 in vitro can prime strong in vivo $CD8^+$ T cell immunity when injected into mice that are deficient in $CD4^+$ T cells (Ridge et al., 1998). Moreover, $CD8^+$ T cell responses to cell-associated antigens depend on $CD4^+$ T cell help (Bennett et al., 1997). This requirement can be overcome in mice depleted of $CD4^+$ T cells by anti-CD40 activating antibody treatment (Bennett et al., 1998; Schoenberger et al., 1998). Besides the CD40-dependent mechanism, it has been suggested that $CD4^+$ T cells may also provide help in a CD40-independent manner (Lu et al., 2000). One such way could be by IL-2 production, which directly enhances $CD8^+$ T cell immunity (Wilson and Livingstone, 2008).

Help from $CD4^+$ T cells during infections is not always necessary during primary $CD8^+$ T cell immunity. However, it is essential for establishing optimal levels of memory and secondary immunity. This observation is based on studies using various infection models, including IAV, LCMV and LM (Belz et al., 2002; Janssen et al., 2003; Shedlock et al., 2003). Importantly, it has been shown that lack of $CD4^+$ T cell help during primary $CD8^+$ T cell priming, but not during the rechallenge phase, directly reduces the activation of memory cells (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). Two models explaining this $CD4^+$ T cell requirement during priming for secondary responses have been put forward. Firstly, $CD4^+$ T cells may be involved in 'programming' $CD8^+$ T cells such that they can reactivate better during secondary challenge (Janssen et al., 2003; Janssen et al., 2005). Alternatively, $CD4^+$ T cells may allow better maintenance of memory $CD8^+$ T cells over time (Sun et al., 2004). However, the type of pathogens examined clearly influences the dependence on $CD4^+$ T cell help. For instance, $CD4^+$ T cell help is needed in both primary and secondary responses during VACV infection for optimal $CD8^+$ T cell immunity (Novy et al., 2007). Experimental models can also affect the outcome, as indicated by the opposite conclusions drawn from experiments using MHC-II deficient mice and

wildtype mice depleted of CD4 T cells with anti-CD4 antibody during LM infection (Sun and Bevan, 2003; Marzo et al., 2004).

1.6.2 Functions of $CD8^+$ effector T cells to control infections

After naive $CD8^+$ T cells have proliferated and differentiated into effector cells, their expression of LN-homing receptors, such as CD62L, is reduced, allowing the cells to migrate out from the LNs (Weninger et al., 2001; Calzascia et al., 2005; Liu et al., 2006b). At the same time, expression of multiple chemokine receptors that promote periphery entry and retention are induced on these activated T cells, and the type of receptors up-regulated is influenced by the route and type of infection and immunisation (Weninger et al., 2001; Calzascia et al., 2005; Liu et al., 2006b; Sandoval et al., 2013). Once an activated T cell recognises its cognate peptide/MHC-I expressed on the infected cell surface, they can exert their effector functions, including the killing of infected cells and the release of cytokines and chemokines.

1.6.2.1 Killing of infected cells

The capacity of activated $CD8^+$ T cell to kill infected cells has long been recognised and this forms the basis of the classical in vitro cytotoxicity assay which has been used to measure the magnitude of $CD8^+$ T cell responses since the 1960s (Brunner et al., 1968; Marker and Volkert, 1973). $CD8^+$ T cells kill infected cells by inducing their apoptosis through two main pathways, namely the perforin/granzyme pathway and the Fas-Fas ligand pathway (Kagi et al., 1994; Lowin et al., 1994).

Expression of perforin and several types of granzymes is induced in activated $CD8^+$ T cells and these molecules are located in secretory vesicles that are known as lytic granules (Garcia-Sanz et al., 1987; Garcia-Sanz et al., 1990; Ebnet et al., 1991; Peters et al., 1991). Multiple studies have demonstrated that perforin and granzymes work together to induce apoptosis of infected cells, in which perforin promotes the transport of granzymes into the target cells by several possible models (Podack et al., 1985; Podack et al., 1988; Metkar et al., 2002; Keefe et al., 2005; Raja et al., 2005; Thiery et al., 2011). Once in the cytoplasm, granzymes,

especially the well-studied granzymes A and B, trigger apoptosis in the infected cells by caspase-dependent and independent pathways (Hayes et al., 1989; Heusel et al., 1994; Talanian et al., 1997; Andrade et al., 1998; Beresford et al., 1999; Goping et al., 2003; Martinvalet et al., 2008). Mice that are deficient in perforin or granzymes show increased pathogenic burdens in multiple infection models (Kägi et al., 1994; Kagi et al., 1994; Walsh et al., 1994; Müllbacher et al., 1996; Müllbacher et al., 1999; Pereira et al., 2000; Balkow et al., 2001; Gupta et al., 2005). These molecules can be produced by other cell types, such as natural killer (NK) cells. However, using an adoptive transfer strategy, it was later directly shown that this pathway mediated by $CD8^+$ T cells is indeed necessary to control infections (Topham et al., 1997; White and Harty, 1998; White et al., 1999; Messingham et al., 2003; Shrestha et al., 2006).

Interaction between Fas on target cells with Fas ligand on activated $CD8^+$ T cells is another major pathway to induce apoptosis in target cells (Trauth et al., 1989; Itoh et al., 1991; Suda et al., 1993; Lynch et al., 1994). Fas ligand expression on $CD8^+$ T cells is enhanced after activation (Suda et al., 1995; Suzuki and Fink, 2000). Fas surface expression can be enhanced on target cells after recognition by antigen-specific $CD8^+$ T cells (Simon et al., 2000; Müllbacher et al., 2002), in the presence of IFN-γ (Xu et al., 1998) or when they become infected (Takizawa et al., 1995; Shrestha and Diamond, 2007). It has been shown that the Fas-Fas ligand pathway is required for providing protection against LM and IAV infection (Topham et al., 1997; Jensen et al., 1998). In contrast, this apoptosis-inducing mechanism seems to play a minor role in controlling other commonly studied infections, including LCMV, VACV and ectromelia virus (Kägi et al., 1995; Müllbacher, 2003).

$CD8^+$ T cells can also kill infected cells through another pathway that is mediated by tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). Binding of TRAIL death receptors expressed on infected cells by TRAIL triggers apoptosis of the infected cells (Wiley et al., 1995; Sedger et al., 1999; Kotelkin et al., 2003; Ishikawa et al., 2005). The role of TRAIL in $CD8^+$ T cell immunity was demonstrated by adoptive transfer experiments, which showed that TRAIL-deficient $CD8^+$ T cells provided less protection and increased viral burden in

recipient mice compared with those received wildtype $CD8^+$ T cells during IAV and West Nile virus infection (Brincks et al., 2008; Shrestha et al., 2012). TRAIL is not expressed exclusively on $CD8^+$ T cells, so other cells may also contribute to the killing of target cells via this pathway (Smyth et al., 2001; Dorothée et al., 2002).

1.6.2.2 Release of cytokines and chemokines

Besides their cytolytic capacity, effector $CD8^+$ T cells can also produce a range of cytokines, including IFN-γ and TNF-α, to control infections. These cytokines are not constitutively expressed in activated $CD8^+$ T cells, instead their expression is induced upon TCR-peptide/MHC-I engagement (Slifka et al., 1999; Slifka and Whitton, 2000b). These cytokines have direct anti-pathogenic effects, inhibiting pathogen replication in vitro and in vivo (Ulker and Samuel, 1985; Schofield et al., 1987; Karupiah et al., 1993; Campen, 1994; Harris et al., 1995; Guidotti et al., 1996; Guidotti and Chisari, 2000; Janssen et al., 2002; Ostler et al., 2002; Biermer et al., 2003). In addition, they can facilitate the functions of other immune cell types, for example by activating macrophages and DCs to control infections (Buchmeier and Schreiber, 1985; Suzuki et al., 1988; Leenen et al., 1994; Harty and Bevant, 1995; White and Harty, 1998; Trevejo et al., 2001). Direct involvement of $CD8^+$ T cell help to other immune cells via cytokine production has been suggested during LM infection where $CD8^+$ T cells can promote the function of macrophages for bacterial clearance (Berg et al., 2005). These cytokines can also facilitate the killing of target cells. IFN-γ has been shown to increase MHC-I expression on infected cells in vivo, which is essential for their elimination during infection with adenovirus (Yang et al., 1995) while TNF-α can bind to its receptors on infected target cells to trigger apoptosis (Holler et al., 1992; Tartaglia et al., 1993; Wohlleber et al., 2012). Activated $CD8^+$ T cells also release chemokines or aid chemokine release by other cell types in peripheral sites, which helps to attract other immune cells and orchestrate the overall immune response (Kim et al., 1998; Wagner et al., 1998; Cook et al., 1999; Zhao et al., 2000; Kakimi et al., 2001).

Overall, $CD8^+$ effector T cells help to control infection during a primary response via multiple pathways that are triggered by the TCR signalling pathway. The direct interaction between an infected cell and an activated $CD8^+$ T cell has been visualised in vitro and in vivo (Stinchcombe et al., 2001; McGavern et al., 2002; O'Keefe and Gajewski, 2005; Barcia et al., 2006; Barcia et al., 2008; Sanderson et al., 2012). The recognition of peptide/MHC-I on infected cells by an activated $CD8^+$ T cell causes relocation of surface TCR and various TCR signalling molecules, such as Lck, to the contact site to form an immunological synapse with the infected cell (Stinchcombe et al., 2001; Barcia et al., 2008). This interaction allows targeted release of effector molecules, including perforin, granzymes and cytokines, from the $CD8^+$ T cells directly to the interacting infected cell (Stinchcombe et al., 2001; McGavern et al., 2002; O'Keefe and Gajewski, 2005; Barcia et al., 2008). All these functions of $CD8^+$ effector T cells are important and work together to control infections.

1.6.3 Memory $CD8^+$ T cell immunity

After the primary $CD8^+$ T cell response, the population size of the activated $CD8^+$ T cells contracts as the majority of the effector cells are eliminated. Although the contraction period generally occurs when the infection starts to be cleared, the duration of antigen availability and the rate of infection clearance do not necessarily influence the kinetics of the contraction phase, as shown in LM and acute LCMV infection models (Badovinac et al., 2002). However, the contraction phase can be influenced by inflammation during early T cell priming (Badovinac et al., 2004). Only memory cells will survive the contraction phase, and these cells are functionally superior to naive cells as they can be activated faster and produce a stronger immune response in the presence of a lower antigen level (Pihlgren et al., 1996; Curtsinger et al., 1998).

In most acute infections, pathogens are cleared after the primary response and so there should be no antigen available for memory immunity maintenance. Indeed, antigens and MHC-I expression are not required for the maintenance of the memory $CD8^+$ T cells (Hou et al., 1994; Lau et al., 1994; Tanchot et al., 1997; Murali-Krishna et al., 1999). However, similar to naive $CD8^+$ T cells, cytokines,

including IL-7 and IL-15, are required for the homeostasis of memory cells in the periphery (Lodolce et al., 1998; Zhang et al., 1998b; Kennedy et al., 2000; Schluns et al., 2000; Burkett et al., 2003; Kaech et al., 2003).

1.6.3.1 Memory precursors

Memory precursor effector cells are formed alongside terminally-differentiated effector cells from naive T cells during $CD8^+$ T cell priming (Jacob and Baltimore, 1999; Opferman et al., 1999). Several factors have been shown to affect the differentiation of $CD8^+$ T cells into these two subsets, including the extent of cell proliferation, the level and duration of TCR stimulation, and the strength of the inflammatory signal during priming (Joshi et al., 2007; Kaech and Wherry, 2007; Sarkar et al., 2008; Obar and Lefrançois, 2010). The two effector subsets can be identified and isolated based on IL-7 receptor expression together with the use of other functional markers, such as the LN-homing receptor CD62L and the senescence marker killer cell lectin-like receptor G1 (KLRG1) (Kaech et al., 2003; Huster et al., 2004; Joshi et al., 2007; Sarkar et al., 2008). Generally, terminally-differentiated effector cells are identified as $CD62L^{low\ (lo)}$ $KLRG1^{high\ (hi)}$ $IL\text{-}7R^{lo}$, while memory precursor cells are $CD62L^{hi}$ $KLRG1^{lo}$ $IL\text{-}7R^{hi}$. These two subsets of effector cells seem to share similar effector functionality and the only major difference is that the terminally-differentiated effector cells are less able to produce IL-2 and have reduced survival and proliferation ability than memory precursor cells (Voehringer et al., 2001; Joshi et al., 2007; Sarkar et al., 2008).

1.6.3.2 Memory $CD8^+$ T cell subsets

Circulating memory $CD8^+$ T cells can be grouped into two major subsets, as defined by the expression of LN-homing receptors CCR7 and CD62L (Sallusto et al., 1999; Wherry et al., 2003b; Huster et al., 2004). They are known as central-memory $CD8^+$ T cells (with a $CCR7^{hi}$ $CD62L^{hi}$ phenotype) and effector-memory $CD8^+$ T cells ($CCR7^{lo}$ $CD62L^{lo}$). These subsets differ in their ability to proliferate during secondary challenge (Unsoeld et al., 2002; Wherry et al., 2003b). Wherry et al (2003b) demonstrated that the adoptive transfer of central-memory $CD8^+$ T cells into mice allows quicker clearance of pathogens after systemic LM, LCMV

and VACV infections than mice transferred with effector-memory T cells. Enhanced protection by central-memory T cells against LCMV has been confirmed in another study (Nolz and Harty, 2011). However, a different study found that effector-memory $CD8^+$ T cells allow better protection than central-memory T cells against VACV infection (Bachmann et al., 2005). The investigators speculated that this inconsistency between their study and Wherry et al (2003b) might be explained by the difference in number of cells transferred between their experiments (Bachmann et al., 2005). Using a VSV intranasal (i.n.) infection model, it was shown that effector-memory $CD8^+$ T cells dominate the induced responses in the adoptively transferred recipients compared to central-memory T cells (Roberts and Woodland, 2004). In contrast, the opposite result was observed in an intravenous (i.v.) Sendai virus infection model (Klonowski et al., 2006). This implies that the types of infections influence the ability of various memory T cell subsets to respond to secondary infection.

Besides the circulating memory cell subsets, memory T cells known as tissue-resident memory cells can be maintained within peripheral tissues (Marshall et al., 2001; Masopust et al., 2001). These tissues include skin (Gebhardt et al., 2009; Liu et al., 2010), intestine (Masopust et al., 2010; Casey et al., 2012), and lung (Salek-Ardakani et al., 2011b; Wakim et al., 2013), as well as the central and peripheral nervous systems (Wakim et al., 2008; Wakim et al., 2010). Inflammation at the peripheral sites, but not the presence of antigens, is important for the recruitment of activated $CD8^+$ T cells and formation of this memory population (Casey et al., 2012; Mackay et al., 2012a). Skin-resident memory $CD8^+$ T cells formed during dermal infection with HSV or VACV provide better protection against subsequent dermal viral challenges compared to the circulating-memory T cells (Gebhardt et al., 2009; Liu et al., 2010; Jiang et al., 2012; Mackay et al., 2012b). This superior protection by tissue-resident memory T cells during local challenges can be explained by their proximity to the site of infection for a rapid secondary response (Ariotti et al., 2012), higher cytotoxicity than those memory cells found in secondary lymphoid organs (Masopust et al., 2001), and ability to rapidly recruit circuiting memory $CD8^+$ T cells (Schenkel et al., 2013).

In addition to the memory cells, effector-like cells that are IL7R^{lo} KLRG-1^{hi} are found to persist into the memory phase, although they disappear over time (Bachmann et al., 2005; Masopust et al., 2006; Hikono et al., 2007). These cells express high levels of granzyme B and it has been demonstrated that they can participate in controlling viral infection against LM, LCMV and VACV infection based on adoptive transfer experiments (Bachmann et al., 2005; Olson et al., 2013). In contrast to these findings, Laouar et al. (2008) suggested that central-memory T cells, but not the effector-like cells, are essential to clear VACV infection. This disparity is probably because of the differences in their experimental setups, including the length of resting period between adoptive transfer of sorted CD8$^+$ T cells and infection challenge in the recipients, as a longer waiting period would favour the survival of central-memory T cells over effector-like cells.

Overall, it is reasonable to suggest that all subsets of memory CD8$^+$ T cells are involved in providing protective immunity during secondary infections. The involvement of individual subsets depends on the pathogen.

1.6.4 CD8$^+$ T cell exhaustion after chronic infection

Some types of chronic infections, in which antigens are available constantly, can lead to CD8$^+$ T cell exhaustion and result in a reduced responsiveness by T cells. LCMV is commonly used as a chronic infection model because virus strains that induce acute and chronic infections in mice have been identified and characterised (Ahmed et al., 1984; Matloubian et al., 1993). Compared to acute infection where LCMV can be effectively cleared, chronic LCMV infection results in a reduction of antigen-specific CD8$^+$ T cells during the primary response phase and progressive loss of cytokine production and cytolytic ability, and a failure to proliferate during subsequent challenge (Moskophidis et al., 1993; Wherry et al., 2003a; Wherry et al., 2005; Wherry et al., 2007; Utzschneider et al., 2013). A similar loss of functionality of CD8$^+$ T cells has been reported from patients infected with human immunodeficiency virus (HIV) or hepatitis C virus (Appay et al., 2000; Goepfert et al., 2000; Gruener et al., 2001). It has been suggested that the high antigen dose available during chronic LCMV infection promotes

exhaustion of $CD8^+$ T cells over time (Mueller and Ahmed, 2009). The importance of antigen persistence in inducing this exhaustion stage in $CD8^+$ T cells is supported with another infection model, where mice were immunised with IAV twice a week for 4 consecutive weeks (Bucks et al., 2009). Exhausted $CD8^+$ T cells expressed high level of a surface protein called programmed cell death 1 (PD-1), which is commonly used as an exhaustion marker (Urbani et al., 2006, Barber et al., 2006, Wherry et al., 2007, Day et al., 2006). This molecule inhibits T cell migration and functions by interacting with PD-1 Ligand 1 and Ligand 2, which can be expressed on many immune cell types during chronic infections (Ishida et al., 1992, Freeman et al., 2000, Latchman et al., 2001, Zinselmeyer et al., 2013, Blackburn et al., 2010).

It is important to note that chronic infection does not always lead to T cell exhaustion. During murine cytomegalovirus infection where the virus can persist in the salivary glands, the induced $CD8^+$ T cells retain a similar level of cytokine production after stimulation during acute and chronic phases of infections (Cavanaugh et al., 2003; Munks et al., 2006). Similarly, functional $CD8^+$ T cells can be found in mice chronically infected with mouse polyomavirus (Lukacher et al., 1999; Wilson et al., 2011). In addition, some viruses, such as HSV and murine γ-herpesvirus 68, can enter a latency stage within infected cells where they remain dormant until reactivation (Walz et al., 1974; Sunil-Chandra et al., 1992). $CD8^+$ T cells induced during infection with this type of virus in mice remain poly-functional and can further proliferate (Freeman et al., 2010; Mackay et al., 2012b). It is speculated that only a low level of antigen is produced from a small percentage of the latently-infected cell population during this type of infection and so $CD8^+$ T cells are not exhausted (Wherry and Ahmed, 2004).

1.7 Antigen presentation on MHC-I

Professional APCs utilise two mechanisms to present peptides on MHC-I, namely direct presentation and cross presentation. Components of these two presentation pathways can affect the immunogenicity of a peptide. The mechanisms involved in antigen processing and presentation via these two pathways are discussed here.

1.7.1 Direct presentation

In direct presentation, antigens are expressed and processed endogenously within APCs to generate peptides for presentation on MHC-I. Early studies on anti-viral $CD8^+$ T cell immunity frequently used virus-infected cells as the target cells to measure the cytotoxicity function of the activated $CD8^+$ T cells isolated from immunised mice (Marker and Volkert, 1973; Zinkernagel and Doherty, 1973). Studies using DNA transfection or recombinant VACV infection to drive expression of antigens of interest in the target cells directly demonstrated that antigens expressed endogenously within the cytoplasm of APCs are utilised for MHC-I presentation (Gooding and O'Connell, 1983; Bennink et al., 1984; Townsend et al., 1984; Townsend et al., 1985; Townsend et al., 1986a). The molecular mechanism for antigen processing and peptide presentation in this pathway is illustrated in Figure 1-2.

Proteasomes play an important role in processing antigens into peptides that can bind to MHC-I (Rock et al., 1994; Harding et al., 1995; Craiu et al., 1997). Proteasomes can generate peptides of various lengths, including peptides of the same length as mature eptiopes or as peptide precursors with extensions at the amino-terminus (Cascio et al., 2001). They are also essential for defining the carboxyl-termini of many presented peptides (Beninga et al., 1998; Mo et al., 1999). Several proteasomal subunits can be induced in APCs after exposure to stimulants (e.g. IFN-γ) to form a variant known as the immunoproteasome with altered cleavage specificity (Morel et al., 2000; van Hall et al., 2000; Chen et al., 2001b). The amino-terminal-extended peptides generated by proteasomes can be further trimmed by cytosolic aminopeptidases, such as tripeptidyl peptidase II (Reits et al., 2004), leucine aminopeptidase (Beninga et al., 1998), puromycin-sensitive aminopeptidase and bleomycin hydrolase (Stoltze et al., 2000). However, it should be noted that peptide liberation from antigens is not perfect. Proteasomes and these peptidases do not always liberate peptides from antigens effectively and they can cleave and destroy the antigenic peptides in some cases (Del Val et al., 1991; Niedermann et al., 1995; Antón et al., 1998; Luckey et al., 1998; Preta et al., 2008; Shen et al., 2011). Proteasomal-independent antigen processing has also been described and it involves many of the proteases mentioned above (López and Del Val, 1997; Seifert et al., 2003; Guil et al., 2006).

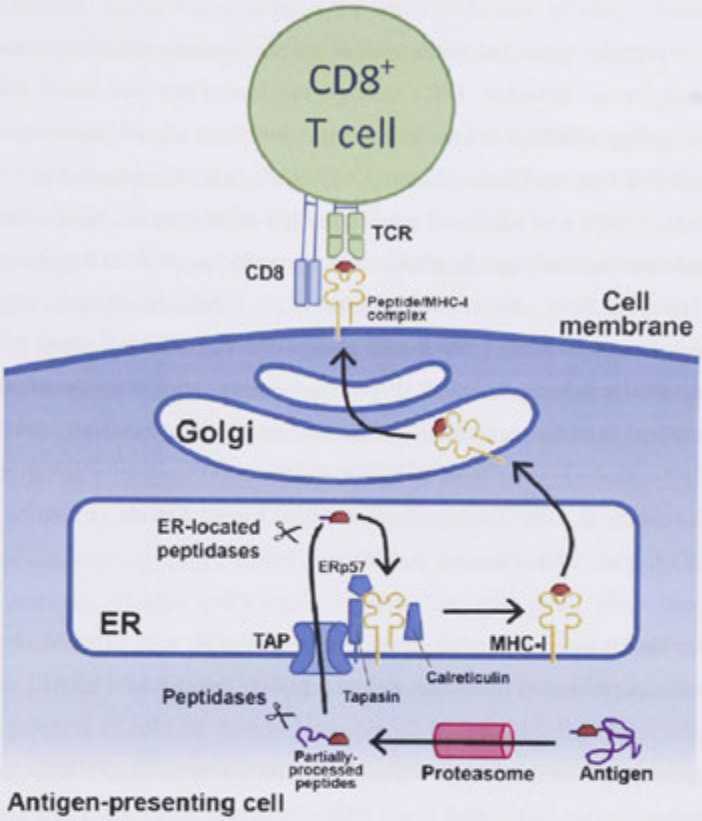


Figure 1-2| Components involved in the direct MHC-I presentation pathway. Cytosolic antigens expressed within APCs are degraded by the proteasome to generate partially-processed peptides. These peptides can be further trimmed by other cytosolic peptidases. They are then translocated into the ER via TAP. Within the ER, peptides can be further processed into appropriate lengths by the ER-located peptidases and are loaded onto MHC-I. This loading process is facilitated by several chaperones, including tapasin and calreticulin. The peptide/MHC-I complexes are then transported to the surface of the presenting cell via the Golgi apparatus. The peptide/MHC-I complexes presented on the cell surface can be recognised by TCRs expressed on $CD8^+$ T cells via an antigen-specific manner.

How an antigen is processed for presentation depends on its physical form and this will be discussed in Section 1.7.4.

The peptides generated in the cytosol are then transported into the lumen of the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP), a heterodimer formed from two related proteins called TAP1 and TAP2 on the ER membrane (Kelly et al., 1992; Kleijmeer et al., 1992; Spies et al., 1992). TAP-mediated peptide transfer requires ATP (Neefjes et al., 1993) and the length of peptides translocated normally ranges from 8 to 13 amino acids (Momburg et al., 1994a; Schumacher et al., 1994). In addition, peptides with certain sequences are more readily transported via TAP than others (Momburg et al., 1994b; Schumacher et al., 1994). Importantly, it has been identified that some minimal peptides presented on MHC-I do not transport efficiently into the ER via TAP unless they are flanked by extra amino acid residues (Neisig et al., 1995; Lauvau et al., 1999), supporting a role for further peptide processing in the ER (York et al., 2006, Hearn et al., 2009).

Amino-terminal extended peptides in the ER are trimmed by the ER aminopeptidases (ERAP) into mature peptides. Two ERAPs, ERAP1 and ERAP2, are expressed in humans while mice only express an ERAP1 homolog, called ER aminopeptidase associated with antigen processing (ERAAP; Saric et al., 2002; Serwold et al., 2002; York et al., 2002; Saveanu et al., 2005). These proteases can process antigenic peptide precursors into minimal peptides that are 8 to 9 amino acids long. It has been suggested that peptide precursors need to bind to MHC-I such that the MHC-I could act as a template to allow ERAPs to trim peptides to their optimal length and prevent complete peptide degradation (Kanaseki et al., 2006). However, it is well demonstrated that minimal antigenic peptides can be generated from amino-terminal extended precursors by purified ERAP molecules alone in vitro (Saric et al., 2002, Serwold et al., 2002, York et al., 2002, Saveanu et al., 2005). In addition, ERAP1 prefers substrates that are 9 to 16 residues long and has minimal activity against shorter peptides (Chang et al., 2005; Nguyen et al., 2011). These data imply that amino-terminal extended peptides are processed in the ER lumen by ERAPs before MHC-I presentation. A carboxyl dipeptidase located in the ER, called angiotensin-converting enzyme, can also remove extra

amino acid residues from the carboxyl-terminus of extended precursors that are generated by proteasomes (Eisenlohr et al., 1992a; Shen et al., 2011). However, its role is likely to be minor because the carboxyl-termini of many examined peptides are defined by cytosolic proteasomes as discussed above.

The processed peptides in the ER are then loaded onto the peptide-binding site of MHC-I. Multiple proteins, including TAP, tapasin, calreticulin and ERp57, are associated with the empty MHC-I/β_2m complex in the ER and form a peptide-loading complex to help stabilise the empty MHC-I and facilitate the peptide loading process (Sadasivan et al., 1996; Ortmann et al., 1997; Hughes and Cresswell, 1998; Gao et al., 2002). Tapasin links MHC-I and TAP together and is required for assembling the peptide-loading complex (Sadasivan et al., 1996; Suh et al., 1999). The stability of the peptide-loaded MHC-I in cells is reduced in the absence of tapasin, suggesting a role of tapasin in facilitating peptides with high affinity to bind to MHC-I (Garbi et al., 2000; Tan et al., 2002; Williams et al., 2002; Howarth et al., 2004). ERp57 is another protein found in the peptide-loading complex (Hughes and Cresswell, 1998; Lindquist et al., 1998; Morrice and Powis, 1998). This protein can bind to tapasin to form a dimer which facilitates peptide loading onto MHC-I and also promotes the replacement of low-affinity peptides from MHC-I with those that have higher affinity (Wearsch and Cresswell, 2007). Calreticulin is another chaperone that plays a role in loading optimal peptides onto MHC-I, but knockout studies show that it is not essential for formation of functional peptide-loading complexes (Gao et al., 2002)

Most short peptides are very unstable and are degraded within seconds in the cytoplasm (Reits et al., 2003). In contrast, some peptides may bind to chaperones, such as heat shock proteins (HSPs), to facilitate direct presentation in certain scenarios. Knocking down expression or functional inhibition of cytosolic chaperone HSP90α can reduce direct presentation (Kunisawa and Shastri, 2006; Callahan et al., 2008). Moreover, the ER-located chaperone gp96 may be involved in delivering precursor peptides to calreticulin after TAP-mediated translocation (Kropp et al., 2010). The interactions between chaperones and peptides cannot be too strong as this would prevent the transfer of peptides onto MHC-I.

After peptide binding to MHC-I, the loaded MHC-I complex dissociates from the peptide-loading complex (Ortmann et al., 1994; Suh et al., 1994; Li et al., 1999). The peptide/MHC-I complex is then transferred onto the cell surface via the Golgi apparatus (Nuchtern et al., 1989; Yewdell and Bennink, 1989; Cox et al., 1990).

Secreted and membrane antigens generated in the ER can be transferred back into the cytoplasm before being processed by the antigen processing mechanism mentioned above (Hammond et al., 1995; Skipper et al., 1996; Bacik et al., 1997; Mosse et al., 1998; Selby et al., 1999). The retrotranslocation of the ER-targeted antigens may be facilitated by the ER-associated protein degradation (ERAD) pathway (Meusser et al., 2005). Other pathways are also involved in generating peptides from these antigens. ER-targeted antigens can be processed directly by proteasomes within the cytoplasm without first being translocated into the ER (Schlosser et al., 2007). Alternatively, some other secreted and membrane proteins have been found to be proteolysed exclusively in the secretory pathway for direct MHC-I presentation. This processing pathway can involve ER-located signal peptidases and signal peptide peptidases (Henderson et al., 1992; Wei and Cresswell, 1992; El Hage et al., 2008), as well as furin, a protease which is mainly located in the trans-Golgi network (Gil-Torregrosa et al., 1998; Gil-Torregrosa et al., 2000; Medina et al., 2009). In these scenarios, the antigen presentation will be TAP-independent.

1.7.2 Cross presentation of exogenous antigens

Exogenous antigens can also be presented on MHC-I and this pathway is called cross presentation (Heath et al., 1998). Bevan (1976b; 1976a) first discovered that $CD8^+$ T cell responses against minor histocompatibility antigens restricted to one MHC-I haplotype could be induced in vivo by cells carrying the same antigens but that had a different MHC-I haplotype. This phenomenon is known as cross priming. Since then, more cross-presented antigens have been identified, including soluble antigens (Staerz et al., 1987), cells artificially loaded with stable antigens (Carbone and Bevan, 1990), virus and protozoan antigens (Jin et al., 1988; Denkers et al., 1993), and tumour cells (Huang et al., 1994). This process is

particularly important for eliciting $CD8^+$ T cell responses against pathogens that do not infect professional APCs.

It is largely agreed that DCs are the major cell type that presents exogenous antigens in vivo (Kurts et al., 2001; Jung et al., 2002; Joffre et al., 2012). In particular, LN-resident $CD8\alpha^+$ DCs (equivalent to human $CD141^+$ DCs (Bachem et al., 2010)) and the closely-related migratory $CD103^+$ DCs are the major cross-presenting subsets (den Haan et al., 2000; Bedoui et al., 2009; Henri et al., 2010; Desch et al., 2011). Other immune cell types, such as $CD8\alpha^-$ DCs (den Haan and Bevan, 2002; McDonnell et al., 2010), plasmacytoid DCs (Mouriès et al., 2008), macrophages (Asano et al., 2011), B cells (Hon et al., 2005) and neutrophils (Beauvillain et al., 2007), may also be involved in cross presentation in some circumstances. Although many DC subsets can capture exogenous antigens effectively, their ability to process these antigens for MHC-I presentation varies and this is the major factor that determines their cross presentation capacity (Schnorrer et al., 2006). Several receptors have been shown to be involved in cross presentation, including DEC-205 (Jiang et al., 1995; Bonifaz et al., 2004), mannose receptor (Burgdorf et al., 2007) and C-type lectin domain Family 9 member A (Clec9A, also known as DNGR-1; Sancho et al., 2009; Zhang et al., 2012). It has been show that different forms of antigens are taken up by different types of endocytic receptors (Burgdorf et al., 2006). In addition, multiple pathways for presenting exogenous antigens on MHC-I have been proposed and studied. They are discussed below and illustrated in Figure 1-3.

1.7.2.1 Phagosome-to-cytosol pathway

In the phagosome-to-cytosol pathway, exogenous antigens are captured into phagosomes from the extracellular space and are exported into the cytoplasm for processing into peptides for MHC-I presentation (Figure 1-3A). This mechanism was first reported by two studies, which demonstrated that exogenous antigens can gain access to the cytoplasm and that the proteasomal degradation of exogenous antigens, but not lysosomal proteolysis, is essential for presenting exogenous antigens (Kovacsovics-Bankowski and Rock, 1995, Norbury et al., 1995). This mechanism requires active capture of exogenous antigen sources into

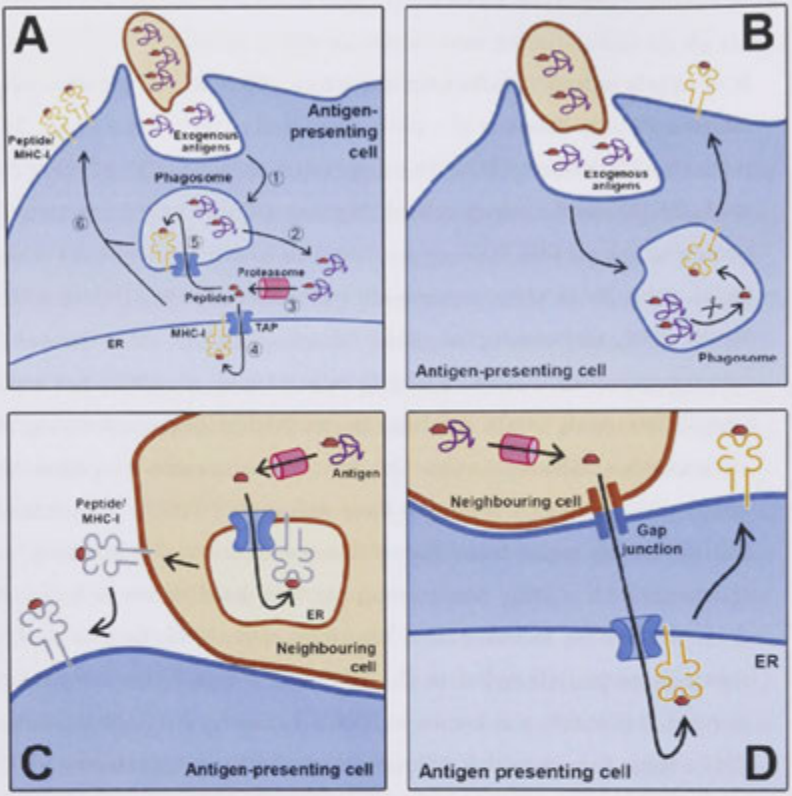


Figure 1-3| Cross presentation pathways. Peptides originated from external antigens can be cross presented onto MHC-I by several possible mechanisms, including the phagosome-to-cytosol pathway (A), vacuolar pathway (B), cross-dressing of peptide/MHC-I (C), and peptide transfer via a gap junction (D). (A) In the phagosome-to-cytosol pathway, antigens are captured into phagosomes (1). The captured antigens are translocated into the cytoplasm for proteasomal processing (2-3). Peptides generated are then translocated into ER (4) or back to the phagosomes (5) for MHC-I binding via TAP. The peptide/MHC-I complexes are then presented on the cell surface (6). (B) In vacuolar pathway, the proteolytic processing of internalised antigens and the peptide loading onto MHC-I occur within the phagosomes exclusively. (C) During cross dressing, peptide/MHC-I complexes presented on neighbouring cells are transferred to the surface of APCs. (D) Peptides generated from the neighbouring cells can also be translocated into APCs via gap junctions for MHC-I loading and presentation.

the phagosomes (Shen et al., 1997) and the TAP-mediated transport of processed peptides from the cytoplasm into the ER for binding onto MHC-I (Kovacsovics-Bankowski and Rock, 1995; Ackerman et al., 2005). In addition to phagocytosis, micropinocytosis is also involved in cross presentation, especially for capturing soluble antigens (Norbury et al., 1995).

Degradation of captured antigens within phagosomes is detrimental to this cross presentation pathway as suggested by a range of evidence. Antigens susceptible to lysosomal proteolysis are less likely to be cross presented via this pathway (Delamarre et al., 2005). In addition, the export of phagocytosed antigens to the cytoplasm and their cross presentation can be enhanced by treating presenting cells with chloroquine or ammonium chloride, which inhibit lysosomal acidification and reduce the activity of several lysosomal proteases (Accapezzato et al., 2005). This is further supported by findings from Amigorena and colleagues (2006; 2009) which showed that DCs have a mechanism to prevent over- acidification of their phagosomes, which would otherwise increase proteolysis of the captured antigens and lead to a reduced level of cross presentation. However, unfolding of exogenous proteins carrying disulfide bonds by IFN-γ-inducible lysosomal thiolreductase, as well as partial processing of antigens within phagosomes by proteases, is necessary for the effective cross presentation of the HSV-1 glycoprotein B (gB) antigen via the phagosomes-to-cytosol pathway (Singh and Cresswell, 2010). Overall, these results demonstrate that a precise control of the degradation of exogenous antigens is essential for this pathway.

The phagocytosed antigens need to gain access to the cytoplasm for proteasomal processing and the ERAD pathway may play an important role here (Ackerman et al., 2006). Sec61 protein, an ER-located channel complex required for retrotranslocation of proteins from the ER into the cytosol for ERAD (Wiertz et al., 1996), can be found in the phagosomes (Guermonprez et al., 2003; Houde et al., 2003). Sec61, together with ATPase p97 which provides energy for ERAD-associated retrotranslocation, are required for this cross presentation pathway (Ackerman et al., 2006).

In addition to the ER, phagosomes can also serve as sites for peptide loading onto MHC-I. Phagosomes isolated from DCs and macrophages have been found to carry all the required machinery for antigen processing, including MHC-I, TAP and peptide-loading complex proteins (Ackerman et al., 2003; Guermonprez et al., 2003), as well as the proteasomes which are associated with the cytosolic side of the phagosomal membrane (Houde et al., 2003). Peptides can be readily transported into the lumen of the purified phagosomes via TAP, where they bind MHC-I (Ackerman et al., 2003, Guermonprez et al., 2003). Burgdorf et al. (2008) reported that early endosomes can provide exogenous antigens to the cytosol for antigen processing and act as a compartment for MHC-I loading, similar to the phagosomes described above. The presence of the ER proteins required for MHC-I presentation within the phagosomes suggests the existence of ER-phagosome fusion, although one report has failed to detect such a process (Touret et al., 2005). It was later identified that Sec22b, a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein that controls membrane fusion, is important for recruiting ER proteins, such as TAP, tapasin and calreticulin, to phagosomes (Cebrian et al., 2011). Knocking down this protein with short hairpin ribonucleic acid (RNA) significantly reduced the cross presentation of exogenous antigens in DCs (Cebrian et al., 2011). If amino-terminal-extended peptides are imported into phagsomes, they may be further processed into final mature peptides (Saveanu et al., 2009).

1.7.2.2 Vacuolar pathway

Processing and presentation of phagocytosed antigens can occur entirely within the phagosomes and this mechanism is known as the vacuolar pathway (Figure 1-3B; Rock, 1996). Pfeifer, et al (1993) and Harding and Song (1994) demonstrated that MHC-I presentation of antigens expressed from vacuolar bacteria or associated with latex beads captured by macrophages did not require newly synthesised MHC-I and that peptide binding to MHC-I did not occur in the ER. The investigators proposed that antigen processing and peptide binding can happen entirely within the vacuolar compartments. It was later reported that treating macrophages with proteasomal inhibitors did not affect the presentation of these antigens (Song and Harding, 1996) and that this pathway does not require

transportation of peptides into the ER (Bachmann et al., 1995). Other exogenous antigens can also be presented via this pathway, including those from protozoa (Bertholet et al., 2006), IAV (Di Pucchio et al., 2008), measles virus (Grommé et al., 1999), inactivated Sendai virus (Liu et al., 1995; Liu et al., 1997), and virus-like particles (Schirmbeck et al., 1995; Ruedl et al., 2002; Leclerc et al., 2007).

Out of the possible proteases in the phagosomal and endosomal compartments, Shen et al (2004) found that the cysteine protease cathepsin S alone could process the full-length model antigen ovalbumin (OVA) into the minimal $CD8^+$ T cell epitope peptide (OVA-257; amino acid sequence SIINFEKL; Falk et al., 1991b; Rötzschke et al., 1991) in vitro. They also demonstrated that cathepsin S plays a key role in priming $CD8^+$ T cell responses against antigens that are cross presented via the vacuolar pathway in vivo (Shen et al., 2004).

1.7.2.3 Cross-dressing: Transfer of peptide/MHC-I complexes

The transfer of membrane proteins between different immune cells has long been observed (Sharrow et al., 1981; reviewed in Davis, 2007). It has been shown that DCs can receive membrane components from other DCs, macrophages, B cells and even T cells during co-incubation in vitro (Harshyne et al., 2001). More importantly, DCs can acquire intact peptide/MHC-I complexes from other cells for presentation to $CD8^+$ T cells (Russo et al., 2000; Herrera et al., 2004) and this route for antigen presentation is known as cross-dressing (Figure 1-3C; Yewdell and Haeryfar, 2005).

The significance of this mechanism for priming was first demonstrated when it was shown that peptide/MHC-I complexes transferred to recipient DCs were able to sustain $CD8^+$ T cell proliferation in vitro (Smyth et al., 2008). It has been suggested that exosomes from donor DCs can facilitate peptide/MHC-I transfer (André et al., 2004). However, others have demonstrated that direct cell contact is essential for this process in vitro (Dolan et al., 2006; Wakim and Bevan, 2011). When a large number of donor cells was transferred into recipient mice, host DCs could acquire peptide/MHC-I complexes to allow proliferation of naive $CD8^+$ T cells and induce their effector functions (Dolan et al., 2006; Smyth et al., 2012) .

Using a DNA vaccination strategy in which a DNA construct encoded a single-chain peptide-MHC-I-β_2m molecule, Li et al. (2012) showed that the single-chain peptide/MHC-I molecules expressed on keratinocytes could transfer to DCs and allow activation of naive and memory $CD8^+$ T cells in vivo. However, in a more physiological setting, Wakim and Bevan (2011) discovered that cross-dressed DCs carrying peptide/MHC-I complexes from parenchymal cells of mice infected with VSV could re-stimulate memory, but not prime naive, $CD8^+$ T cell responses. The difference between these in vivo findings may reflect the number of donor cells and the amount of peptide/MHC-I available for transfer.

1.7.2.4 Peptide transfer via gap junction

Gap junctions are intercellular channels that allow molecules to be transferred between connected cells (Goodenough and Paul, 2009). Neijssen et al (2005) first discovered that expressing connexin 43, a major component of gap junctions, in tumour cells allowed antigenic peptides to be transferred from the tumour cells to DCs for antigen presentation in vitro (Figure 1-3D). Processed peptides from apoptotic tumour cells and antigen-loaded DCs can also be transferred into DCs via gap junctions for antigen presentation in vitro (Mendoza-Naranjo et al., 2007; Pang et al., 2009). A recent study showed that immunising mice with *Salmonella*-infected tumour cells which expressed an increased level of connexin 43 allows an induction of a $CD8^+$ T cell response to control distal tumour formation (Saccheri et al., 2010). Although this presentation mechanism has been examined using various tumour models, all these systems involved either transduction of the connexin 43 gene or in vitro stimulation to enhance connexin 43 expression in the antigen donor cells. Its physiological role in vivo has yet to be demonstrated.

1.7.3 Contributions of different cross presentation pathways

The availability of the many possible cross presentation pathways clearly demonstrates the importance of this process for inducing $CD8^+$ T cell responses in the host and contradicts Zinkernagel (2002), who suggested that cross presentation and cross priming are not physiologically relevant. The relative contribution of the different cross presentation mechanisms to the induction of $CD8^+$ T cell immunity depends on various factors. Firstly, the nature of the

antigen influences how the antigen will be processed. For instance, OVA attached to iron oxide particles is only cross presented via the phagosome-to-cytosol pathway, while OVA incorporated with poly(lactic-co-glycolic acid) can be processed by the vacuolar pathway (Shen et al., 2004). Moreover, the size of the captured antigens affects how they are processed (Mant et al., 2012). Secondly, certain presenting cells may favour particular pathways for presenting exogenous antigens. For example, an in vitro study has indicated that $CD8\alpha^-$ DCs, but not $CD8\alpha^+$ dendritic cells, prefer peptide/MHC-I transfer over conventional cross presentation (Smyth et al., 2008).

It should be noted that only a few studies have analysed their contributions to prime $CD8^+$ T cells in vivo. Using TAP-deficient bone marrow chimeric mice, it has been demonstrated that in vivo cross priming of $CD8^+$ T cells against antigens from tumours and polio virus is completely reliant on TAP expression in the bone marrow derived APCs, suggesting the importance of the phagosome-to-cytosol pathway (Huang et al., 1996; Sigal et al., 1999). Shen et al (2004) used a similar system with TAP- and cathepsin S-double-deficient bone marrow chimeric mice to dissect the contributions of phagosome-to-cytosol and vacuolar pathways for cross priming $CD8^+$ T cell immunity. They discovered that although both pathways contribute to cross priming of $CD8^+$ T cell against cell-associated antigens, the phagosome-to-cytosol pathway seems to be more important for particulate antigens and influenza A virus infection in vivo (Shen et al., 2004).

1.7.4 Substrates for direct and cross presentation

Stable proteins available in the cytoplasm can be processed by proteasomes for direct presentation (Moore et al., 1988). However, it is generally accepted that the major substrates for generating antigenic peptides are newly-synthesised gene products that are rapidly-degraded by proteasomes (Reits et al., 2000; Schubert et al., 2000; Princiotta et al., 2003; Donohue et al., 2006; Qian et al., 2006; Dolan et al., 2010; Dolan et al., 2011; Croft et al., 2013). Defective ribosomal products (DRiPs) have been demonstrated to be one such substrate form (Yewdell et al., 1996; Princiotta et al., 2003; Qian et al., 2006; Dolan et al., 2010; Dolan et al., 2011).

Physiological antigen processing from full-length proteins is relatively inefficient: on average only one peptide/MHC-I complex is formed from 2000 degraded proteins (Princiotta et al., 2003). This problem can be overcome experimentally. Firstly, the efficiency of direct presentation can be enhanced by increasing the degradation of antigens by proteasomes (Townsend et al., 1988; Princiotta et al., 2003). Secondly, minimal peptide sequences, known as minigenes, can be directly expressed in the cytosol with a start codon or in the ER with an ER-targeted signal sequence at the amino-terminus (Anderson et al., 1991; Bacik et al., 1994). These minigene constructs minimise the requirement for proteolytic processing and, with ER-targeting, bypass TAP translocation, allowing the peptides to be presented more efficiently (Bacik et al., 1994; Antón et al., 1997; Porgador et al., 1997). For instance, it has been found that APCs infected with VACV expressing full-length OVA express around 10-fold fewer OVA-257/H-2K^b complexes on the cell surface than APCs infected with VACV expressing OVA-257 minigene ($3x10^3$ compared to $3.5x10^4$ OVA-257/H-2K^b molecules after a six-hour infection; Princiotta et al., 2003).

In contrast, cross-presented antigens need stability to survive long enough to be acquired by antigen-presenting cells. It is proposed that substrates for cross priming exist either as **(a)** processed peptides that are complexed and stabilised by chaperones, such as HSPs (Srivastava, 2002), or **(b)** stable proteins (Yewdell and Haeryfar, 2005). Partially-degraded antigens have also been found to be the source of cross presentation (Serna et al., 2003; Blachère et al., 2005), but their role has not been further examined.

Multiple HSPs isolated from tumour cells, including ER-resident HSP gp96 (also known as GRP94), and cytosolic gp70 and HSP90, have been shown to induce anti-tumour immunity in vivo (Srivastava et al., 1986; Ullrich et al., 1986; Udono and Srivastava, 1994). It was later found that antigenic peptides complexed with HSPs can be cross presented onto MHC-I in vitro by macrophage and DCs (Suto and Srivastava, 1995; Basu et al., 2000). In addition, CD8$^+$ T cell immunity can be induced in mice immunised with HSPs either isolated from antigen-expressing cells (Arnold et al., 1995; Suto and Srivastava, 1995; Casey et al., 2003) or in

vitro complexed with peptides (Blachere et al., 1997; Mo et al., 2011). Various antigen delivery systems based on this HSP/peptide model have been developed and suggested as potential cancer vaccines (Suzue et al., 1997; Yamazaki et al., 1999; Oizumi et al., 2007).

The role of HSP/peptide complexes for cross presentation under physiological conditions has been examined mainly by Srivastava and colleagues. Using lysates of MHC-I-mismatched antigen-expressing cells as immunogens in vivo, Binder and Srivastava (2005) showed that cross priming was suppressed when all HSPs of interest, but not stable antigens, were depleted. Purified HSPs from antigen-expressing cells induced $CD8^+$ T cell immunity in the absence of any stable antigens (Binder et al., 2007). In addition, the same research group showed that antigen-loaded cells lost their cross presentation ability after being treated with specific HSP90 inhibitors (Callahan et al., 2008). Expression of HSP90 and gp96 within antigen-donor cells can improve cross presentation of a stable antigen, although the mechanism responsible was not examined (Basta et al., 2005).

Even though these studies support the role of HSP/peptide complexes in cross priming, their importance remains highly questionable. The first source of uncertainty is the specificity of peptide binding to HSPs. Several studies have attempted to isolate and characterise the peptides chaperoned by HSPs. Two separate studies showed that an extended antigenic peptide precursor from an engineered OVA antigen, but not the final antigenic OVA-257 peptide, could associate with HSP90 and gp96 isolated from antigen-expressing cells (Kunisawa and Shastri, 2006; Binder et al., 2007). Furthermore, viral and tumour antigenic fragments associated with HSPs have been identified from virus-infected or tumour cells (Nieland et al., 1996; Ishii et al., 1999; Meng et al., 2001; Grossmann et al., 2004). However, other studies failed to recover antigenic peptides from purified HSP (Paz et al., 1999; Lev et al., 2008). More importantly, short peptides diffuse freely within the cytosol and nucleus and are degraded rapidly within cells, suggesting that the majority of peptides are not stabilised by chaperones to any significant level (Reits et al., 2003). It is estimated that only up to 0.4% of the gp96 chaperones are associated with peptides in vivo (Demine and Walden, 2005). Further, the affinity and stability between gp96 and an examined antigenic

peptide derived from VSV nucleoprotein (NP) was found to be weak at physiological temperature and pH (Ying and Flatmark, 2006). These studies support the model in which HSPs interact weakly with peptides to help harbour them between important antigen processing machineries (Kunisawa and Shastri, 2006). However, HSP/peptide interactions should not be stable enough for cross presentation.

Several studies have also questioned the role of HSP/peptide complexes in eliciting antigen-specific immunity. It has been shown that injecting mice with gp96-expressing fibroblasts can induce anti-tumour immunity, independent of the antigen availability (Baker-LePain et al., 2002). This non-specific induction of immunity may be due to the adjuvant property of the HSPs (Baker-LePain et al., 2002; Vabulas et al., 2002). In support of this hypothesis, an engineered form of gp96 can improve the in vivo cross presentation of unchaperoned OVA antigen (Oizumi et al., 2007). However, it has also been suggested that the observed adjuvant effect could be caused by contamination of the purified HSPs with other potent adjuvants, such as lipopolysaccharides (Nicchitta, 2003; Tsan and Gao, 2004; Yewdell, 2005).

In contrast to the HSP/peptide model, multiple studies have clearly demonstrated that cross-presented antigens are acquired by antigen-presenting cells in the form of stable antigens. Firstly, unlike Binder and Srivastava (2005), Shen and Rock (2004) showed that removal of the stable OVA proteins from cell lysate resulted in a failure to induce a $CD8^+$ T cell response against the dominant OVA-257 peptide via cross presentation. Secondly, Norbury et al (2004) demonstrated that the OVA-257 peptide expressed from a rapidly-degraded antigen or as a short-lived minigene construct failed to be cross-presented in vivo, in spite of the high level of the OVA-257 peptide generated from these unstable antigens (Princiotta et al., 2003). They further demonstrated that stable OVA constructs were cross-presented effectively (Norbury et al., 2004). Thirdly, Wolkers and colleagues (2004) found that peptides within the rapidly-cleaved ER-inserting sequence of an antigen were significantly less immunogenic than the same peptides derived from the stable part of the same protein during cross presentation in vivo, although peptides from either position were effectively directly presented in vitro. Fourthly,

Janda et al (2004) found that cross presentation of antigens from LM-infected cells by dendritic cells was enhanced by inhibiting the proteasomal-mediated antigen degradation pathway in infected cells that no longer expressed new antigens. Fifthly, a DNA vaccine encoding a stable antigen induced a better $CD8^+$ T cell response in vivo than one encoding a rapidly-degraded antigen when examined in a setting where only cross presentation was allowed (Bins et al., 2007). Although cross presentation of an accumulated stable antigen was improved by HSP expression, the rapidly-degraded version of the same antigen failed to be cross presented even in the conditions favouring HSP expression (Basta et al., 2005). According to the HSP/peptide theory, one would assume that increasing production of peptides would increase the amount of HSP-associated substrates for cross presentation. However, the studies mentioned above clearly reject this hypothesis and suggest that stable antigens are the natural substrates for cross presentation.

Altogether, these results suggest that cross-presented peptides on MHC-I originate from stable antigens. Consistent with these findings, a study has shown that minigene constructs of two IAV $CD8^+$ T cell epitopes were not cross-presented to prime $CD8^+$ T cells in vivo when expressed from VACV recombinants (Lev et al., 2008). Surprisingly, the same study found that another $CD8^+$ T cell epitope from IAV, known as PA-224 from the acid polymerase (PA), expressed as a minigene could be cross-presented from VACV-infected cells. Additional data suggested that there was a stable cytosolic PA-224 peptide pool within the infected cells, possibly with indirect association with HSP90 (Lev et al., 2008). The effect of this HSP on cross priming of the full-length PA antigen was not examined. In contrast to the PA-224 peptide, expression of HSP90 has minimal effect on the cross presentation of native VACV antigens from VACV-infected cells (Lev et al., 2008). Similarly, the ER-located gp96 is not involved in cross priming against VACV and IAV antigens (Lev et al., 2009). These results suggest that exceptions like PA-224 aside, minimal peptides are not normally protected by HSPs extensively and are not used as substrates for cross presentation.

1.8 Factors influencing immunogenicity of a particular antigen

Although many antigens can be expressed from a virus, only a few of them can elicit $CD8^+$ T cell responses. Furthermore, the size of the response induced against individual peptides varies, with some dominating the overall response while the contribution of other is at the limit of detection (Yewdell and Bennink, 1999). This phenomenon, known as immunodominance, has been observed in mice during DNA immunisations (Rodriguez et al., 2002; Liu et al., 2006a; Riedl et al., 2009) and infections with various types of pathogens, including large T antigen from simian virus 40 (Mylin et al., 2000), IAV (Chen et al., 2000; Belz et al., 2001), LCMV (van der Most et al., 1996), VACV (Tscharke et al., 2005; Moutaftsi et al., 2006), HSV-1 (St. Leger et al., 2011), and LM (Busch and Pamer, 1998). A similar phenomenon has been observed in humans during infections and vaccinations (Yu et al., 2002a; Smith et al., 2005b; Wu et al., 2011). During immunisation with recombinant vaccines, one would hope that the induced immunity can focus on the recombinant antigens that are artificially expressed from the vaccines, but not on the vaccine vector itself. Thus it is important to understand how immunogenicity can be manipulated to favour antigens of interest. Factors influencing immunogenicity can be divided into two categories: **(a)** factors related to antigen presentation and **(b)** factors related to the $CD8^+$ T cell repertoire.

1.8.1 Factors related to the presented antigen

1.8.1.1 Expression kinetics of the antigen

It is generally accepted that antigens expressed early and at high levels during infections or immunisations are more immunogenic than those expressed late and in lower abundance. During VACV infections, $CD8^+$ T cell immunity tends to target native viral proteins that are expressed early during infection (Oseroff et al., 2005; Tscharke et al., 2005; Moutaftsi et al., 2006; Oseroff et al., 2008; Moutaftsi et al., 2010). Similarly, anti-HSV-I immunity is skewed towards early-expressed genes (St. Leger et al., 2011). One possible explanation brought forward by these studies is that early gene products are more available for presentation by DCs (Oseroff et al., 2008; St. Leger et al., 2011). It should be noted that this correlation is not absolute, with some late gene products being immunogenic (Moutaftsi et

al., 2006; Oseroff et al., 2008; St. Leger et al., 2011), implying that other factors also contribute to the immunogenicity of antigens.

Studies that have directly compared the same antigenic constructs expressed by promoters with different kinetics also lead to similar conclusions. Recombinant antigens expressed from a late promoter are less immunogenic than the same antigens expressed from an early promoter when expressed from VACV, as shown in two virus strains (Coupar et al., 1986; Kastenmuller et al., 2007). Enhancing expression during early infection is also important to further improve the immunogenicity of antigens (Coupar et al., 1986; Baur et al., 2010). A similar result has been observed for adenovirus immunisation, where modifying an immediate-early promoter to enhance expression level improves the immunogenicity of the inserted antigen (Sridhar et al., 2008).

1.8.1.2 Efficiency of antigen processing and presentation

As shown in Section 1.7.4, the efficiency of antigen processing during direct presentation affects the surface expression level of the peptide/MHC-I complex. Therefore, it also influences the immunogenicity of a given peptide when direct priming is the main mechanism to induce $CD8^+$ T cell immunity. During VACV infection, the efficiency of peptide generation from full-length antigens for direct presentation can be influenced by having different flanking sequences and this in turn affects the immunogenicity of antigens expressed (Del Val et al., 1991). Further, antigenic constructs that enhance direct presentation, including rapidly-degraded proteasomal substrates and minigenes (Townsend et al., 1988; Porgador et al., 1997; Princiotta et al., 2003), generally induce better $CD8^+$ T cell responses than the full-length proteins when expressed from VACV (Lawson et al., 1994; Restifo et al., 1995; Deng et al., 1997; Tobery and Siliciano, 1997). As discussed in Section 1.7.4, translocation of a processed peptide into the ER via TAP also affects the efficiency of the peptide binding to MHC-I. Minigene constructs tagged with an ER-targeting sequence at their amino-termini have been designed to avoid this bottleneck to improve immunogenicity of a given antigen (Restifo et al., 1995; Fu et al., 1998). These data indicate that efficiently processed antigens are more likely to be highly immunogenic.

If cross presentation is the preferred pathway for priming $CD8^+$ T cells during a particular type of vaccination, rapid degradation of antigens will be detrimental to the level of immunogenicity elicited, as discussed in Section 1.7.4. This has been demonstrated during intramuscular and intradermal (i.d.) DNA immunisations (Wong and Pamer, 2001; Bins et al., 2007; Schliehe et al., 2012), which involves cross presentation of antigens sourced from transduced muscle cells (Fu et al., 1997; Cho et al., 2001). Increasing the stability of peptide vaccines by extending them with three amino acids at the amino-terminus allows for more effective cross priming of $CD8^+$ T cells in vivo (Wei and Sherman, 2007). Furthermore, modifications to specifically target exogenous antigens to the cross presentation pathway have been developed to enhance $CD8^+$ T cell cross priming. This mainly involves the use of antibodies to specifically target antigens to endocytic receptors important for cross presentation, which have been mentioned in Section 1.7.2 (Bonifaz et al., 2002; Bonifaz et al., 2004; He et al., 2007; Caminschi et al., 2008; Hesse et al., 2013).

It is important to remember that multiple factors influence the presentation pathway favoured during a vaccination. For instance, although enhanced degradation of an antigen favours its immunogenicity during intramuscular immunisation with a high dose of adenovirus (Rouard et al., 2003), stable full-length antigens are more favourable when a lower dose of virus is used (Millar et al., 2007). This clearly suggests that the dose of an immunogen influences the optimal form of antigens. Likewise, increasing instability of antigens to enhance immunogenicity during DNA immunisation has also been reported (Rodriguez et al., 1997), clearly contradicting the reports discussed above (Wong and Pamer, 2001; Bins et al., 2007; Schliehe et al., 2012). As the antigen, the dose and the immunisation regime used were different amongst these studies, one may conclude that these factors affect how an antigen is presented during DNA immunisation.

1.8.1.3 Peptide binding affinity to MHC-I

Once a peptide is processed successfully, its MHC-I binding affinity affects how long it is presented on the surface of APCs and could in turn affect immunogenicity. Many highly immunogenic peptides have a relatively high

MHC-I binding affinity when determined as the MHC-I association rate (Sette et al., 1994; Kotturi et al., 2008). Furthermore, peptides with too low binding affinity fail to present on the cell surface and do not induce any immune responses during peptide immunisation (Sette et al., 1994). Indeed, bioinformatic prediction of MHC-I binding affinity has been used as the first step to identify possible epitope candidates within a virus (e.g. Zhong et al., 2003; Moutaftsi et al., 2006). However, highly immunogenic peptides do not necessarily have the highest MHC-I binding affinity. For example, the highly immunogenic $CD8^+$ T cell epitope from the IAV NP antigen during IAV infection in BALB/c mice, known as NP-147, has a relatively low MHC-I affinity compared with other IAV peptides (Chen et al., 2000). Some investigators have suggested that a slow MHC-I dissociation rate is more important because it relates directly to the stability of a peptide/MHC-I complex and its longevity at the cell surface (Ojcius et al., 1993; Harndahl et al., 2012). In support of this idea, Burg and colleagues (1996) showed that most mapped $CD8^+$ T cell epitopes they tested had a low MHC-I dissociation rate.

To enhance affinity and stability of peptide binding to MHC-I, peptide-MHC-I-β_2m single chain trimers and similar technology have been developed (Mottez et al., 1995; Yu et al., 2002b; Palmowski et al., 2009; Li et al., 2012). These constructs improve antigen presentation and the ability to induce $CD8^+$ T cell responses in vivo. Alternatively, in some experimental models where the natural peptides have a modest MHC-I affinity, immunising mice with modified peptides with enhanced MHC-I binding affinity or stability can improve the $CD8^+$ T cell responses against the natural peptides (Sarobe et al., 1998; van Stipdonk et al., 2009; Watson et al., 2012). However, one needs to be aware that immunising mice with modified peptides with too high MHC-I affinity can result in reduced quality of $CD8^+$ T cell immunity (McMahan et al., 2006). This may be due to an expansion of $CD8^+$ T cells that recognise peptide/MHC-I poorly or subversion of T cells into an anergic state (McMahan et al., 2006). Overall, while peptides need to bind onto MHC-I to induce a $CD8^+$ T cell response, this property alone cannot account for the immunogenicity of a given antigen.

1.8.2 Factors related to the CD8$^+$ T cell repertoire

Other than the importance of antigen presentation, several characteristics of CD8$^+$ T cells specific to a given peptide/MHC-I complex also modulate the induced CD8$^+$ T cell response.

1.8.2.1 Naive CD8$^+$ T cell precursor frequency

The naive CD8$^+$ T cell precursor frequency is the number of CD8$^+$ T cells in a non-immune individual that can recognise a given peptide. There is a diverse population of naive CD8$^+$ T cells in each individual and only few of them can recognise a particular peptide. Previously, only indirect and time-consuming methods could estimate a naive CD8$^+$ T cell precursor frequency. One common method used was a limiting dilution analysis based on either in vitro cytotoxicity assay with naive cells or in vivo proliferation and expansion of transferred T cells relative to the host's endogenous response (Lindahl and Wilson, 1977; Blattman et al., 2002; Choi et al., 2002; Seedhom et al., 2009). Recently, a new strategy that allows direct quantification of the naive epitope-specific CD8$^+$ T cell has been developed: it involves labelling naive cells from all secondary lymphoid organs with peptide/MHC-I tetramers, followed by magnetic enrichment of the labelled cells (Kotturi et al., 2008; Obar et al., 2008).

With this isolation technique, several studies have shown that peptides recognised by more naive CD8$^+$ T cells tend to induce higher responses in several infection models (Kotturi et al., 2008; Obar et al., 2008; Tan et al., 2011). Similarly, lowered immunogenicity of several VACV antigens in C57Bl/6 x DBA/2 F1 progeny mice compared to the parental strains correlates with reduced numbers of antigen-specific naive CD8$^+$ T cells in the F1 mice (Flesch et al., 2010). In contrast, La Gruta et al (2010) found that the immunogenicity of individual IAV peptides during infection in C57Bl/6 mice does not correlate to the naive cell precursor frequency. Rather, they demonstrated that effective T cell recruitment and expansion are more important in their IAV infection model (La Gruta et al., 2010).

1.8.2.2 TCR binding affinity for peptide/MHC-I

TCR affinity indicates how well a given TCR can bind to the peptide/MHC-I complex it recognises. This can either be measured in vitro with purified TCR and peptide/MHC-I complexes (Corr et al., 1994) or estimated from the association of $CD8^+$ T cells to peptide/MHC-I tetramers ex vivo (Kalergis et al., 2001). $CD8^+$ T cells with low affinity to peptide/MHC-I are less likely to become completely activated (Kalergis et al., 2001). Zehn et al (2009) studied the effects of TCR affinity in vivo using LCMV recombinants to express OVA-257 peptide variants which bind equally well to MHC-I H-$2K^b$ but are recognised by TCR-transgenic OT-I $CD8^+$ T cells with different affinity. They found that although OT-I cells can proliferate during infection with LCMV expressing low affinity TCR ligands, the period of expansion is significantly reduced compared to the well-recognised peptide variants (Zehn et al., 2009). During an IAV infection, $CD8^+$ T cells specific to the dominant NP-366 peptide express TCRs with lower affinity to the cognate peptide compared with those specific to the co-dominant PA-244 peptide (La Gruta et al., 2004). This correlates with the inferior ability of the NP-366-specific $CD8^+$ T cells to produce cytokines (La Gruta et al., 2004).

However, $CD8^+$ T cell function cannot be further improved once the TCR affinity reaches to a certain level, suggesting a ceiling effect (Schmid et al., 2010). In addition, TCRs with too high affinity can be detrimental to T cell function (Kalergis et al., 2001). It is hypothesised that $CD8^+$ T cells carrying TCRs with too high affinity are less likely to make serial engagement with peptide/MHC-I on APCs, which is essential for ideal T cell activation (Valitutti et al., 1995; Hudrisier et al., 1998; Kalergis et al., 2001). On the other hand, $CD8^+$ T cells expressing TCRs with a lower affinity can form memory T cells that respond during challenge (Zehn et al., 2009). These results indicate that there is a fine balance in TCR affinity for a strong immunogenicity.

1.8.2.3 Functional avidity of $CD8^+$ T cells

Besides the individual affinity of TCR to peptide/MHC-I, other factors also affect the overall sensitivity of a $CD8^+$ T cell to respond to its cognate epitope, and collectively this is known as functional avidity (Slifka and Whitton, 2001).

Increased functional avidity of $CD8^+$ T cells improves their ability to respond when the level of antigen is low (Slifka and Whitton, 2001; Mothe et al., 2012). For instance, HIV-infected patients who successfully control the infection carry $CD8^+$ T cells that have a higher functional avidity than those that fail to control the infection, as measured by the ability of T cells to respond to low antigen concentration and to various antigenic variants (Mothe et al., 2012).

Functional avidity is influenced by the following factors. Firstly, the strength of TCR binding will affect how well it can recognise peptide/MHC-I, as discussed in Section 1.8.2.2. Secondly, multiple TCR molecules on the cell surface of activated $CD8^+$ T cells are organised into clusters, which improves the overall avidity to peptide/MHC-I complexes compared to naive T cells (Fahmy et al., 2001). Thirdly, the CD8 co-receptor contributes to the functional avidity as it improves the overall avidity of the TCR-peptide/MHC-I interaction, and therefore allows $CD8^+$ T cells to respond to lower levels of cognate antigens (Cawthon and Alexander-Miller, 2002; Holler and Kranz, 2003; Mothe et al., 2012). Finally, enhanced expression of TCR signalling adaptor molecules, such as Lck, also correlates with better functionality of $CD8^+$ T cells during LCMV and VACV infections (Slifka and Whitton, 2001; Amoah et al., 2012). Overall, increasing functional avidity improves the outcome of immunity during an infection.

1.8.2.4 T cell competition

Competition for presented peptides on APC between naive $CD8^+$ T cells during priming can also affect the responses induced. This model has been used to explain how TCR-transgenic $CD8^+$ T cells can negatively influence the host's endogenous responses specific to the same antigens (Butz and Bevan, 1998; Kedl et al., 2000). This could be caused by the down-regulation of peptide/MHC-I on APC after interacting with T cells (Kedl et al., 2002). T cells with different specificities can also compete with each other because multiple peptides are presented on the same APC during an immunisation (Kedl et al., 2000; Kedl et al., 2002). The availability of a highly immunogenic peptide in a DNA vaccine affects the priming of T cells against a less immunogenic peptide presented on the same constructs during the primary response (Rodriguez et al., 2002; Liu et al., 2006a;

Riedl et al., 2009). Similarly, immunodomination by highly immunogenic antigens has been detected during secondary IAV and VACV infections (Andreansky et al., 2005; Kastenmuller et al., 2007). In addition, the presence of a high number of T cells specific to a particular peptide can suppress responses against other peptides (von Herrath et al., 1994; Chen et al., 2000; Kedl et al., 2000; Kedl et al., 2002; Lin et al., 2013). Importantly, a high affinity $CD8^+$ T cell specific for one peptide can suppress the priming of a low affinity $CD8^+$ T cell with another specificity, probably due to the enhanced activation and proliferation ability of the high affinity T cells (Kedl et al., 2002; Dzutsev et al., 2007). Recently, Lin et al (2013) showed that the immunodomination effect is more profound during primary peripheral VACV infections than systemic infections, probably due to the lower number of APCs available for priming in draining LNs and a competition for co-stimulation betweeen $CD8^+$ T cells. Thus, systemic immunisation enhances the responses against less immunogenic antigens.

1.9 Vaccinia virus

VACV is an *Orthopoxvirus* within the wider *Poxviridae* family (Moss, 2007). It has a linear double-stranded DNA genome (Easterbrook, 1967; McCrea and Lipman, 1967), which is approximately 190,000 base pairs (bp) long (Geshelin and Berns, 1974; Goebel et al., 1990; Meyer et al., 1991; Antoine et al., 1998; Meisinger-Henschel et al., 2007). Within the Orthopoxvirus genus, there are other closely-related species that cause diseases in animals and humans, including ectromelia virus that causes mousepox, variola virus that causes smallpox in humans, as well as cowpox and monkeypox viruses which can cause zoonotic infections in humans. VACV was used as the vaccine to eradicate smallpox in the last century. In addition, VACV is the prototype member of the Poxviridae family and is extensively studied to understand the biology of poxviruses.

The basic form of an infectious VACV mature virion found within infected cells contains two lateral bodies and a biconcave-shaped core carrying the DNA genome, covered with a lipid membrane to form a brick-shaped particle, approximately 200 nm x 300 nm (Peters, 1956; Epstein, 1958; Westwood et al.,

1964; Hollinshead et al., 1999). Some mature virions can be wrapped by a double layer of lipid membrane originated from the Golgi apparatus (Hiller and Weber, 1985). The outer membrane of this form of virion can fuse with the cell membrane to release the virus onto the cell surface or into the extracellular space (Ichihashi et al., 1971). This type of virus particle is known as the enveloped virion (Moss, 2006), and has an additional lipid membrane layer compared to the mature virion.

Infectious viruses can enter cells either by direct viral-cell membrane fusion on the cell surface (Armstrong et al., 1973; Chang and Metz, 1976; Carter et al., 2005) or via macropinocytosis where virions are taken up into vesicles followed by viral-vesicle membrane fusion (Dales, 1963; Dales and Kajioka, 1964; Townsley et al., 2006; Lucas et al., 2008; Mercer and Helenius, 2008; Mercer and Helenius, 2009; Mercer et al., 2010). Viral cores are then released into the cytoplasm where viral gene transcription and viral replication takes place, using viral machinery (Cairns, 1960; Becker and Joklik, 1964; Baroudy and Moss, 1980; Jones and Moss, 1984; Broyles and Moss, 1986; Earl et al., 1986; Ahn et al., 1990). VACV genes can be categorised into early, intermediate and late genes based on the time they are expressed after infection (reviewed; Broyles, 2003; Yang et al., 2010; Yang et al., 2011). More recently, it has been shown that early genes can be separated into two subgroups by cluster analyasis, known as E1.1 and E1.2, in which E1.1 genes are expressed earlier and at an increased level compared to E1.2 genes (Assarsson et al., 2008; Yang et al., 2010). After four to five hours of infection, viral DNA and new virion particles will begin to be produced and packed within discrete areas of the cytoplasm known as viral factories (Cairns, 1960; Dales, 1963). Mature virions can be seen in infected cells six hours after infection (Dales, 1963).

During VACV infection, multiple host cellular pathways are modulated by viral gene products. Firstly, host protein synthesis is inhibited starting from very early during infection (Shatkin, 1963; Becker and Joklik, 1964; Moss, 1968). VACV also encodes proteins to suppress the various anti-viral responses induced within infected cells, which would otherwise inhibit viral gene translation and viral replication (Carroll et al., 1993; Davies et al., 1993; Myskiw et al., 2009). This

includes interfering with multiple pathways important for inducing an anti-viral state within infected cells, including the IFN-I and NF-κB signalling pathways (Gedey et al., 2006; Meng et al., 2009; Myskiw et al., 2009; Fagan-Garcia and Barry, 2011). Although these genes are not all essential for virus growth in general, some of them are required for the virus to replicate in certain cell lines in vitro and they are known as host range genes (Perkus et al., 1990; Beattie et al., 1991; Wyatt et al., 1998; Shisler et al., 1999; Brandt and Jacobs, 2001). Some other non-essential genes, known as immunomodulatory genes, have no apparent effect on normal viral replication in vitro, but are involved in the modulation of immune responses induced during VACV infection and can influence pathogenesis (Alcami and Smith, 1992; Symons et al., 1995; Sroller et al., 2001).

1.9.1 VACV as a vaccine to induce immune responses

Immunity induced by one *Orthopoxvirus* can provide cross-protective immunity against infections with another *Orthopoxvirus* species, and this is the basis for using VACV as a smallpox vaccine. Live cowpox virus was shown by Jenner (1798) to be an effective vaccine against smallpox and was subsequently used in local vaccination programs in the 19th century. However, it was found that the VACV used as the smallpox vaccine in later immunisation programs was different from natural cowpox virus isolated from infected cattle or cattle farmers (Downie, 1939a; Downie, 1939b; Fenner et al., 1988; pp258-271). During the global smallpox eradication program in the mid 20th century, multiple live VACV strains were used throughout the world as smallpox vaccines, including strain New York City Board of Health in the US, strain Lister in Europe, Africa, Asia, as well as strain Tian Tan in China (Fenner et al., 1988; pp581-587). There are differences amongst VACV strains in terms of pathogenicity, but these vaccine strains were considered to be immunogenic as a smallpox vaccine at that time (Fenner et al., 1988; pp581-587).

After VACV immunisation, various adaptive immune responses are induced and immunological memory is maintained. Primary VACV immunisation in humans results in the activation and expansion of VACV-specific $CD8^+$ T cell immunity which peaks at two to four weeks after vaccination, followed by a period of

contraction to form memory $CD8^+$ T cells (Terajima et al., 2003; Rock et al., 2005). The activated $CD8^+$ T cells isolated during the peak of primary response are poly-functional as they are able to produce multiple effector molecules, including granzymes, IFN-γ, TNF-α, and IL-2 (Amara et al., 2004; Rock et al., 2005; Precopio et al., 2007). $CD4^+$ T cells follow a similar trend of expansion, contraction and memory maintenance (Amara et al., 2004). Antibody responses peak at around one month after vaccination in humans and reduce gradually afterwards (Crotty et al., 2003; Frey et al., 2003). More importantly, VACV-specific cellular and humoral immunity from vaccinated people is detectable and significantly higher than in non-immunised people, in some cases even 70 years after a single vaccination (Crotty et al., 2003; Hammarlund et al., 2003).

In order to dissect how individual arms of immunity contribute to controlling VACV infection, animal models are required. The strain of VACV that is widely used in experimental settings is the strain Western Reserve (WR). WR was derived from 18 passages of VACV strain New York City Board of Health in mouse brains (Bronson and Parker, 1941; Parker et al., 1941). This virus is neuropathogenic and more virulent than many other VACV strains, including those used as smallpox vaccines (Parker et al., 1941; Tartaglia et al., 1992; Fang et al., 2005; Hayasaka et al., 2007; Zhang et al., 2010). As a result, this virus has not been used as a smallpox vaccine in humans, but as an experimental tool. Both innate and adaptive immunity are important in controlling WR infection, although their contribution varies depending on the dose and site of infection. During intraperitoneal (i.p.) infection, NK cells are needed to control early viral replication (Bukowski et al., 1983; Martinez et al., 2010b). Monocytes have also been shown to help control virus growth at the inoculation site and virus dissemination to other sites during WR infection (Barbalat et al., 2009; Fischer et al., 2011; Hickman et al., 2013). A new population of innate immune cells, which express neutrophil phenotypic markers ($Ly6C^+Ly6G^+$) but have a morphology similar to monocytes, have been described recently and are important for controlling VACV replication and preventing extensive tissue damage during dermal WR infection (Fischer et al., 2011; Hickman et al., 2013).

Adaptive immunity is also critical for the control of WR infection. RAG-deficient mice, which do not have B or T cells (Mombaerts et al., 1992; Shinkai et al., 1992), do not control WR growth and fail to recover during i.n., i.p. or skin scarification infection (Xu et al., 2004; Mota et al., 2011; Goulding et al., 2012). However, the contribution for each division of adaptive immunity depends on the infection route. $CD4^+$ T cells and B cells are necessary to control primary i.p. WR infection while $CD8^+$ T cells play a minor role, preventing further virus dissemination in mice deficient for $CD4^+$ T and B cells (Xu et al., 2004). On the other hand, mice require an early $CD8^+$ T cell response to protect them from an i.n. infection with a sub-lethal dose of WR (Goulding et al., 2012). Although transfer of serum from WR-immunised mice allows quicker recovery from i.n. infection, depletion or deficiency of $CD4^+$ T cells and B cells does not affect the outcome of pathogenesis compared to wildtype mice (Goulding et al., 2012). Using a different mouse strain and the i.n. model, it has been reported that $CD4^+$ and $CD8^+$ T cells are required synergistically to control WR (Belyakov et al., 2003). For local infection routes, one report failed to identify any significant role of $CD8^+$ T cells in influencing the formation of viral lesions after VACV infection via skin scarification (Spriggs et al., 1992). However, with the use of mutant mice with various degrees of immunodeficiency, a recent report demonstrated that both B and T cells are essential to provide protection against viral replication at the infection site and viral spread into internal organs during skin scarification infection (Mota et al., 2011). In a similar dermal infection model in ear pinnae, Hickman et al (2013) used intravital microscopy and observed that antigen-specific $CD8^+$ T cells eliminate infected monocytes that are moving out from the viral lesions, and they worked cooperatively with $Ly6G^+$ innate immune cells (mentioned above) to resolve lesions.

B cell and T cell memory induced by immunisation with a low dose of WR or attenuated VACV strains is necessary to provide potent protection against challenge with a lethal dose of WR (Belyakov et al., 2003; Wyatt et al., 2004; Liu et al., 2010; Jiang et al., 2012). Furthermore, immunising mice with synthetic VACV $CD8^+$ T cell epitope peptides in the presence of adjuvants or transfer of memory VACV-specific $CD8^+$ T cells into naive mice can improve recovery and survival from VACV challenge (Snyder et al., 2004; Salek-Ardakani et al., 2008;

Moutaftsi et al., 2009; Salek-Ardakani et al., 2011a). These studies demonstrate an important role for $CD8^+$ T cell memory in controlling VACV infection. However, it should be noted that vaccination route, the nature of peptides for immunisation and challenge routes all affect the protection outcome (Moutaftsi et al., 2009; Liu et al., 2010; Jiang et al., 2012 and studies discussed above). Therefore, the immunity generated by VACV and the requirement of individual arms of immunity in different challenge models are not always the same.

The data discussed above indicate that both innate and adaptive immunity work together to control VACV infection. The potent immune response induced during VACV immunisation attracts medical researchers to explore whether this virus can be used to make recombinant vaccines.

1.9.2 VACV as a recombinant vaccine vector

Following the eradication of smallpox, the focus was switched to investigate whether VACV can be used as a vector for vaccines against other diseases. Large DNA fragments can be inserted into VACV stably using a homologous recombination strategy which is relatively straightforward (Mackett et al., 1982; Panicali and Paoletti, 1982; Smith and Moss, 1983). Many experimental vaccines have since been developed and used in animal models (Panicali et al., 1983; Smith et al., 1983a; Smith et al., 1983b; Smith et al., 1984; Schneider et al., 1998; Goonetilleke et al., 2003; Verreck et al., 2009). The first VACV used as a recombinant vaccine on a large scale expresses the rabies virus glycoprotein (Wiktor et al., 1984; Blancou et al., 1986). It is used as a vaccine bait to control rabies in wildlife in Europe and North America (Brochier et al., 1991; Brochier et al., 1995; Hanlon et al., 1998). In addition, some other recombinant VACV are being examined in clinical trials as recombinant vaccines against HIV, tuberculosis and malaria, or as an immunotherapy strategy against cancers (McConkey et al., 2003; McShane et al., 2004; Moorthy et al., 2004; Mwau et al., 2004; Smith et al., 2005a; Webster et al., 2005; Adamina et al., 2010; Currier et al., 2010; Kantoff et al., 2010; Breitbach et al., 2011; Heo et al., 2013; Tameris et al., 2013). These vaccines show different levels of success in clinical trials.

As stated above, VACV strain WR is commonly used in animal models and it is also used widely to generate experimental VACV-based vaccines for examination in mice. However, because of its high virulence, it is not safe for clinical use in humans. In addition, the pathogenicity and potential complications caused by the smallpox vaccines used in the global smallpox eradication program are deemed to constitute a high risk by today's vaccine standards (Fenner et al., 1988; pp581-587). To overcome this problem, attenuated strains of VACV are used as recombinant vaccine vectors. Two commonly used attenuated strains are NYVAC and modified VACV Ankara (MVA). NYVAC was generated by targeted deletion of 18 non-essential genes, including host range and immunomodulatory genes, from VACV strain Copenhagen (Tartaglia et al., 1992). NYVAC has a reduced ability to replicate in many mammalian cell lines and shows low pathogenicity in animal models, including in immunocompromised models (Tartaglia et al., 1992; Edghill-Smith et al., 2003).

MVA was generated by more than 500 serial passages on chicken embryo fibroblasts of a VACV strain originating in Turkey, chorioallantois VACV Ankara (CVA) (Mayr et al., 1975). As a result, MVA has lost its ability to grow on most mammalian cell lines and is completely non-pathogenic and does not replicate in immunocompromised mice and monkeys (Werner et al., 1980; Meyer et al., 1991; Carroll and Moss, 1997; Drexler et al., 1998; Stittelaar et al., 2001; Wyatt et al., 2004). However, this virus remains able to replicate well in chicken embryo fibroblasts and the baby hamster kidney (BHK-21) cell line (Drexler et al., 1998), allowing a high titres of virus to be grown relatively easily. Through genomic mapping and sequencing, it was found that there are six major deletions in the MVA genome, with many other mutations along the genome (Altenburger et al., 1989; Meyer et al., 1991; Antoine et al., 1998; Meisinger-Henschel et al., 2007). Importantly, many host range and immunomodulatory genes are lost in MVA, compared to WR and CVA (Meyer et al., 1991; Antoine et al., 1998; Meisinger-Henschel et al., 2007). These include host range genes C12L and K1L (Gillard et al., 1986; Perkus et al., 1990; Oguiura et al., 1993; Shisler et al., 1999), C3L, which encodes the complement control protein (Kotwal et al., 1990; Isaacs et al., 1992), A52R, M2L and N1L, which inhibit NF-κB activation (Bartlett et al., 2002; Harte et al., 2003; DiPerna et al., 2004; Cooray et al., 2007; Hinthong et al.,

2008), B13R, which inhibits apoptosis (Dobbelstein and Shenk, 1996; Kettle et al., 1997), as well as B8R and B19R which code for the soluble IFN-γ and interferon-I receptors respectively (Alcami and Smith, 1995; Symons et al., 1995; Alcami et al., 2000; Symons et al., 2002). Some of these deleted genes have multiple functions. For instance, the host range gene K1L prevents NF-κB activation and inhibits the IFN-I-induced antiviral signalling pathway (Shisler and Jin, 2004; Meng et al., 2009) while N1L has an anti-apoptotic property within infected cells, in addition to its role in inhibiting NF-κB (Aoyagi et al., 2007; Cooray et al., 2007).

Two recent studies have examined the importance of the large deletions by generating recombinants from CVA and strain Lister with targeted deletions of these genomic regions (Meisinger-Henschel et al., 2010; Dimier et al., 2011). They showed that these deletions only result in moderate virus attenuation (Meisinger-Henschel et al., 2010; Dimier et al., 2011). Importantly, recombinants carrying all of the major deletions can still replicate and cause mild illness in infected mice, especially in those that are immunodeficient (Meisinger-Henschel et al., 2010; Dimier et al., 2011). This clearly indicates that mutated genes outside the major deletions are essential for the complete loss of replicative ability and virulence of MVA in vivo. Indeed, even small in-frame deletions found in some genes from MVA can result in loss of function and can contribute to the avirulence of MVA (McCoy et al., 2010).

Although MVA is highly attenuated, it is able to induce humoral and cellular memory in animals and humans against subsequent challenge with pathogenic poxviruses (Earl et al., 2004; Wyatt et al., 2004; Coulibaly et al., 2005; Parrino et al., 2007; Seaman et al., 2010; Wilck et al., 2010). In addition, MVA has been examined as a recombinant vaccine vector and it has been found that recombinant antigens expressed from MVA are immunogenic and can provide protective immunity (Sutter et al., 1994; Ramírez et al., 2000; Goonetilleke et al., 2003; Ramlau et al., 2008). Some of these vaccines are designed to induce $CD8^+$ T cell immunity (McConkey et al., 2003; Smith et al., 2005a; Webster et al., 2005). As a $CD8^+$ T cell response is also required to control infection of the virulent strain

WR, it is important to dissect and compare how the $CD8^+$ T cell response is elicited during immunisation with VACV strains WR and MVA.

1.9.3 $CD8^+$ T cell priming during immunisation with VACV

The mechanism involved in eliciting $CD8^+$ T cell immunity during VACV infection has mainly been studied using strain WR. Using bone marrow chimeric models, Sigal et al (1999) and Lenz et al (2000) independently showed that bone-marrow derived cells are important for priming anti-VACV $CD8^+$ T cells. Within these cells, macrophages and DCs are the two main cell types that are infected during WR infection in vitro and in vivo (Norbury et al., 2002; Sanchez-Puig et al., 2004; Hickman et al., 2008; Abadie et al., 2009; Hickman et al., 2011b). However, only DCs are responsible for $CD8^+$ T cell priming. Using confocal and multiphoton microscopy to study the draining lymph nodes excised from mice infected with WR in footpads, Norbury et al (2002) found that $CD8^+$ T cells preferentially interact with and form clusters around WR-infected DCs in an antigen-specific manner, but not with infected macrophages. With two photon intravital microscopy to study live mice infected subcutaneously, it was later shown that this clustering of naive $CD8^+$ T cells with DCs infected with WR expressing cognate antigen results in T cell activation (Hickman et al., 2008). In contrast to the stable contacts formed with infected DCs, $CD8^+$ T cells only transiently interact with infected macrophages in the draining lymph nodes (Hickman et al., 2011b). Although this interaction with infected macrophages can be improved by depleting DCs, it does not sustain effective $CD8^+$ T cell priming (Hickman et al., 2011b). Similarly, depleting DCs completely abrogates $CD8^+$ T cell immunity during i.p. MVA infection (Liu et al., 2008). These studies clearly demonstrate that $CD8^+$ T cells are primed by DCs but not macrophages during VACV infection.

To understand which DC subsets are needed for priming anti-VACV $CD8^+$ T cell immunity, most studies have used an in vitro priming assay in which the proliferation level of naive TCR transgenic $CD8^+$ T cells is measured after co-culture with individual DC subsets isolated from infected mice (e.g. Belz et al., 2004). LN-resident $CD8\alpha^+$ DCs were found to be mainly responsible for $CD8^+$ T

cell priming when mice were infected with WR subcutaneously in the footpads or through the i.v. route (Belz et al., 2004; He et al., 2006). A study using i.v. infection also found that $CD8\alpha^+$ DCs play a more important role than $CD8\alpha^-$ DCs for priming one day after infection, although they seem to share a similar $CD8^+$ T cell priming capability at an earlier time point (six hours) post infection (Yammani et al., 2008). On the other hand, it has recently been reported that $CD103^+$ DCs which migrate from the infected lung, but not tissue-resident $CD8\alpha^+$ DCs, are involved in priming an anti-VACV $CD8^+$ T cell response during i.n. infection (Beauchamp et al., 2010). The importance of different subsets of priming DCs between various infection routes might be influenced by a differential ability of the virus to infect individual DC subsets after different routes of infection are used.

Dermal infection of MVA in mice has been studied by Combaddiere and colleagues (Abadie et al., 2009; Duffy et al., 2012; Liard et al., 2012). They firstly found that macrophages and DCs from the draining lymph nodes can present antigens to activate $CD8^+$ T cells (Abadie et al., 2009). Interestingly, purified $CD8\alpha^+$ DCs fail to simulate naive polyclonal $CD8^+$ T cells ex vivo to produce IFN-γ (Abadie et al., 2009). One problem with this assay with polyclonal T cells is that it does not guarantee that the stimulation is antigen-specific and truly represents T cell priming. Liard et al (2012) later showed that infected epidermal Langerhans cells can migrate to draining LNs after mice are immunised with MVA via the dermal route. They also showed that Langerhans cells are involved in $CD8^+$ T cell priming in vivo by using a Langerin-diphtheria toxin receptor transgenic mouse model. As Langerin is not only expressed by Langerhans cells, but also by some dermal DCs, the investigators had made sure that only Langerhans cells were depleted in their experiments (Liard et al., 2012). However, whether $CD8^+$ T cells are directly primed by infected Langerhans cells or via cross priming by other DCs was not determined in the study. More recently, it has been found that the bone marrow can be a priming site for $CD8^+$ T cells and that myeloid DCs in the bone marrow can stimulate polyclonal $CD8^+$ T cells (Duffy et al., 2012). The priming in bone marrow requires transfer of MVA from the inoculation site to the bone marrow by neutrophils (Duffy et al., 2012). Priming activity in the bone marrow seems to be lower than in draining LNs and their

relative contribution to the overall $CD8^+$ T cell response has yet to be directly compared. In addition, further research is needed to determine whether infectious virions or expressed viral proteins are transported by neutrophils for priming in the bone marrow. Therefore, the DC subsets for $CD8^+$ T cell priming during VACV infection have yet to be identified conclusively.

1.9.4 Differences in MHC-I antigen presentation pathways between VACV WR and MVA

Direct presentation is thought to be the key pathway for inducing $CD8^+$ T cell responses during VACV infection (Yewdell and Haeryfar, 2005). This is supported by several studies, although they mainly focused on strain WR only. Some of the evidence for this has been mentioned in various sections in this chapter and they are summarised here. Firstly, many antigens expressed as minigenes or rapidly degraded constructs, which favour direct priming, have similar immunogenicity or are even more immunogenic than stable full-length proteins when expressed by WR (Minev et al., 1994; Restifo et al., 1995; Tobery and Siliciano, 1997). Secondly, two studies have used TAP-deficient mouse models to suggest that direct infection of bone marow-derived APC is important for $CD8^+$ T cell priming (Sigal et al., 1999; Norbury et al., 2001). Thirdly, there is direct visual evidence of naive $CD8^+$ T cells interacting with WR-infected DCs in the draining LNs of infected mice in an antigen-specific manner, leading to T cell activation (Norbury et al., 2002; Hickman et al., 2008; Hickman et al., 2011b). Fourthly, pre-treating mice with a synthetic oligonucleotide containing unmethylated cytosine-guanine dinucleotide motif (CpG), which has been reported to inhibit cross presentation exclusively (Wilson et al., 2006), does not inhibit priming of $CD8^+$ T cell responses during i.p. or subcutaneous WR infections (Xu et al., 2010). These studies support the hypothesis that direct presentation can prime anti-VACV $CD8^+$ T cell immunity.

In some scenarios, responses against VACV antigens may be cross primed. For instance, viral antigens from WR-infected MHC-I-mismatched cells can be cross-primed relatively well in vivo (Larsson et al., 2001; Norbury et al., 2004; Lev et al., 2009). It has also been suggested that cross presentation may be the preferred

pathway for T cell priming during local VACV infection (Shen et al., 2002). In addition, induction of $CD8^+$ T cell responses against some VACV antigens is resistant to direct presentation blockage and responses against these antigens can be effectively cross-primed from infected MHC-I mismatched cells (Basta et al., 2002). It has also been speculated that antigens expressed by late VACV promoters should be exclusively cross presented because infection of DCs is abortive and does not generate late gene products (Bronte et al., 1997; Engelmayer et al., 1999; Subklewe et al., 1999; Chahroudi et al., 2006). Importantly, the ability for a late gene product to be cross presented is reduced if the antigen is not released from viral factories in infected cells, suggesting a role of VACV to modulate cross presentation of late antigens (Tewalt et al., 2009).

While it might seem reasonable to assume that the biology of different VACV strains would be similar, a study has shown that that MVA may differ from WR in the way it elicits $CD8^+$ T cell responses (Gasteiger et al., 2007). Specifically, Gasteiger et al (2007) argued that $CD8^+$ T cells are largely cross-primed during MVA infection. Firstly, infecting DCs in vitro with MVA expressing tyrosinase (Tyr) as a rapidly-degraded antigen enhances the direct presentation ability of its $CD8^+$ T cell epitope, Tyr-369. However, the immunogenicity of this antigen in mice is significantly lower than its stable unmodified counterpart. Similarly, the OVA-257 minigene, which allows better direct presentation, is also less able to prime $CD8^+$ T cell in vivo when expressed from MVA, compared with full-length OVA (Gasteiger et al., 2007). Gasteiger et al (2007) further found that that CpG pre-treatment of mice can significantly inhibit responses against all MVA-expressed antigens, contradicting findings for WR (Xu et al., 2010). With these results, Gasteiger et al (2007) concluded that anti-MVA $CD8^+$ T cell immunity is mainly cross-primed. It was reasoned that the reduced level of maturation and early apoptosis of infected DCs may prevent direct priming in vivo, but favour cross-priming by uninfected DCs (Drillien et al., 2004; Chahroudi et al., 2006; Kastenmuller et al., 2006; Guerra et al., 2007; Pascutti et al., 2011). Another study also suggested an involvement of cross presentation of antigens from infected DCs to induce anti-MVA responses (Liu et al., 2008). Contrary to these studies, interactions between naive $CD8^+$ T cells and MVA-infected DCs have been observed in vivo using intravital microscopy, clearly demonstrating a role of

direct presentation (Kastenmüller et al., 2013). Further, reduction of maturation of DCs following WR infection in vitro has been observed in multiple studies and many of them claimed that cross presentation may be important for priming anti-WR responses (Engelmayer et al., 1999; Subklewe et al., 1999; Jenne et al., 2000; Bonini et al., 2001; Yates and Alexander-Miller, 2007). Despite these arguments, the weight of evidence makes it clear that direct presentation is more important.

In summary, direct priming by WR is now well established as the main mechanism in vivo. For MVA, the priming pathway remains controversial and it requires further examination.

1.10 Aims of this thesis

Our understanding of the presentation pathway for priming $CD8^+$ T cell responses during MVA infection lags behind that of WR substantially. In addition, no direct comparison of $CD8^+$ T cell priming between VACV strains WR and MVA has been reported. Due to the importance of MVA as a recombinant vaccine vector, more in-depth studies are required. Any further information generated would help to improve the design of recombinant MVA vaccines.

This thesis investigates the presentation pathways involved in priming $CD8^+$ T cell responses against antigens expressed from VACV strains WR and MVA through four aims:

a) to dissect the presentation mechanisms involved in $CD8^+$ T cell priming against individual VACV antigens (Chapter 3);

b) to examine the immunogenicity of recombinant antigens that are targeted for direct presentation when expressed from VACV strains WR and MVA (Chapter 4);

c) to investigate why enhancing direct presentation reduces the immunogenicity of a well-characterised IAV antigen expressed from VACV (Chapter 5); and

d) to characterise $CD8^+$ T cell responses elicited against recombinant antigens fused to VACV virion core proteins (Chapter 6).

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Media, buffers and solvents

2.1.1.1 Media

Dulbecco's Modified Eagle Medium (DMEM)

DMEM with high glucose and phenol red (Life Technologies), supplemented with 2 mM L-glutamine (Life Technologies). For some uses, DMEM was further supplemented with 2% (volume/volume (v/v)) or 10% of heat inactivated foetal bovine serum (FBS; Serana, Australia). There were called D2 and D10 respectively. DMEM without FBS was called D0.

FBS, heat inactivated, from Serana, Australia.

Agarose plaque media

1% (weight/volume (w/v)) low melting point agarose (Life Technologies) in D2 media.

Agarose overlay media with 0.04% X-gal

1% (w/v) low melting point agarose in D2 media, supplemented with 0.04% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; BioVectra, Canada).

Carboxymethyl cellulose (CMC) plaque media

0.4% (w/v) of medium viscosity CMC (Sigma-Aldrich) in D2 media.

DC media

D10 media with 10 ng/ml recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems) and 50 μM 2-Mercaptoethanol.

Phenol red-free CMC plaque media

0.4% (w/v) of medium viscosity CMC in DMEM (without phenol red; Life Technologies), supplemented with 2mM L-glutamine and 2% FBS (v/v).

T cell media

D10 media with 50 μM 2-mercaptoethanol (Life Technologies) and 10 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) buffer (Life Technologies).

Luria-Bertani (LB) media

10 g/L tryptone (Bacto), 5 g/L yeast extract (Bacto), 10 g/L sodium chloride (Sigma-Aldrich) in deionised water. It was supplemented with 100 mg/ml ampicillin (Sigma-Aldrich) or 50 mg/ml kanamycin (Sigma-Aldrich) before use.

LB-agar plates

10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride and 15 g/L of Bacto-agar (Bacto) in deionised water. Before pouring into plates, ampicillin or kanamycin was added to warm liquefied LB-agar media to final concentration of 100 μg/ml or 50 μg/ml respectively.

Super optimal broth with catabolite repression (SOC) medium

20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L sodium chloride, 2.5 mM potassium chloride (Sigma-Aldrich), 20 mM glucose (AnalaR) and 10 mM magnesium chloride (Sigma-Aldrich) in deionised water.

2.1.1.2 Buffer

0.5 M Ethylenediaminetetraacetic acid (EDTA), pH 8

0.5 M EDTA in sterile water. pH was adjusted to 8.0 with sodium hydroxide pellets (Sigma-Aldrich).

Magnetic-activated cell sorting (MACS) buffer

0.5% BSA (Sigma-Aldrich) and 2 mM EDTA (pH 8) in PBS.

Phosphate-Buffered Saline (PBS; 1× or 10×)

Obtained from the John Curtin School of Medical Research, the Australian National University, Australia. 1× PBS contains 210 mg/L monopotassium phosphate monobasic, 9 g/L sodium chloride and 726 mg/L disodium phosphate in sterile water.

PBS+2%FBS

1×PBS supplemented with 2% FBS.

Red cell lysis buffer

0.14 M ammonium chloride (Sigma-Aldrich) and 19 mM Tris-Cl in sterile water. pH was adjusted 7.2.

10 mM Tris Buffer, pH 9

10 mM Tris base (Sigma-Aldrich) in sterile water. pH was adjusted to 9.0 with 36% hydrochloric acid (Univar).

1× Tris-acetate-EDTA (TAE) buffer

40 mM Tris base, 20 mM glacial acetic acid (Univar) and 1 mM EDTA (pH 8) in deionised water.

2.1.1.3 solvents

Acetone, from Merck.

Dimethyl sulfoxide (DMSO), from Sigma-Aldrich.

Ethanol, from Merck.

Methanol, from Merck.

Sterile water, from Baxter Healthcare.

2.1.2 Chemicals and Reagents

2.1.2.1 Reagents for molecular biology

100 bp and 1,000 bp DNA markers, from New England Biolabs.

1% agarose for DNA gel electrophoresis

1% (w/v) UltraPure Agarose (Life Technologies) in 1× TAE buffer.

Axyprep Plasmid MiniPrep Kit, from Axygen.

BigDye terminator for DNA sequencing (Life Technologies)

Obtained from the Biomolecular Resource Facility, John Curtin School of Medical Research, the Australian National University.

Bromophenol blue, from Merck.

Competent *Escherichia coli* for plasmid transformation

Competent α-select (Gold Efficiency) *E.coli* cells were obtained from Bioline. Competent *E. coli* strains DH5α and XL10-gold competent cells were also used. These cells were prepared in the Tscharke lab.

Deoxyribonucleotide triphosphate (dNTP) mix, 10 mM, from Bioline

6× DNA gel loading buffer

50% (v/v) glycerol (Sigma-Aldrich), 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol (Sigma-Aldrich) in deionised water. Aternatively 6× DNA gel loading buffer were obtained from New England Biolabs.

DNA staining solution

SYBR Safe DNA gel stain solution (Invitrogen), 1 in 10,000 diluted in deionised water.

Geneclean spin kit, from MP Biomedicals.

In-Fusion HD Cloning Kit, from Clontech.

LigaFast rapid DNA ligation kit, from Promega.

Phusion and *Taq* DNA polymerases, from New England Biolabs.

Potassium chloride, from Sigma-Aldrich, 1M in sterile water.

Restriction enzymes, from New England Biolabs.

Sodium acetate, from AnalaR, 2.4M in sterile water.

Sodium hydroxide, from Sigma-Aldrich, 5M in sterile water.

20% Sodium N-lauroylsarcosinate solution, from Sigma-Aldrich.

Sucrose, from Amresco.

Supercoiled DNA ladder, from Life Technologies.

10× Thermopol reaction buffer

Obtained from New England Biolabs. 1× Thermopol reaction buffer was used in various reactions.

2.1.2.2 Reagents for cell culture and virology

10 mg/ml blasticidin S in HEPES buffer, from InvivoGen.

Crystal violet solution

0.1% (w/v) of crystal violet (Sigma-Aldrich) and 15% (v/v) ethanol in sterile water.

DAB substrate solution

It was prepared by dissolving tablets from SIGMAFAST DAB With Metal Enhancer Tablet Set (Sigma-Aldrich) in sterile water. The solution contains 0.5 mg/ml of 3,3'-Diaminobenzidine tetrahydrochloride (DAB), 0.2 mg/ml cobalt chloride, 0.3m g/ml urea hydrogen peroxide, 0.05 M Tris and 0.15 M sodium chloride.

Lipofectamine 2000, from Life Technologies.

36% Sucrose solution

36% (w/v) sucrose (Amresco) in 10 mM Tris buffer pH 9.

10 μg/ml Proteinase K

10μg/ml of proteinase K (Roche) in 50μM Tris buffer (pH 8).

0.4% trypan blue solution, from Life Technologies.

Trypsin

0.05% (w/v) trypsin with 0.53mM EDTA (Life Technologies) in PBS.

2.1.2.3 Reagents for immunology

4'-Aminomethyltrioxsalen hydrochloride (AMT)

1 mg/ml AMT (Sigma-Aldrich) dissolved in sterile water. The solution was further diluted in PBS before use.

4,5,8-Trimethylpsoralen (TMP)

2.5 mg/ml TMP (Sigma-Aldrich) dissolved in DMSO. The solution was further diluted in PBS before use.

5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE)

1 mg/ml CFSE (1.79mM; Sigma-Aldrich) dissolved in DMSO. It was further diluted in D0 before use.

Brefeldin A

5 mg/ml brefeldin A (Sigma-Aldrich) dissolved in methanol. It was further diluted in D10 before use.

$CD8^+$ T cell isolation kit II (mouse), from Miltenyi Biotec.

DimerX H-$2K^b$:Ig fusion protein, obtained from BD Biosciences.

Horse cytochrome c (cytc)

100 mg/ml horse cytc resuspended in sterile water. It was diluted in PBS to a concentration of 25 mg/ml before injection into mice.

Isoflurane, Inhalation Anaesthetic, from Pharmachem.

Paraformaldehyde, 16% in H_2O, from Electron Microscopy Sciences.

Recombinant mouse IL-2

1 µg/ml recombinant mouse IL-2 (R&D Systems) resuspended in D10.

Saponin

5% (w/v) saponin (Fluka) dissolved in sterile water. It was further diluted in PBS-2%FBS before use.

Vybrant DiD cell-labelling dye, from Life Technologies.

2.1.3 Plasmids

Plasmids used in this thesis were purified as described in Section 2.2.9. They were used as DNA templates for PCR or DNA sequencing reactions (Sections 2.2.1 and 2.2.11) or as a transfer plasmids for generation of VACV recombinants (Sections 2.2.13 and 2.2.14). Table 2-1 shows the plasmids that were available for use in this thesis while Table 2-2 illustrates the plasmids generated in this thesis. Please note that some of the plasmids were generated with the assistance of S.A. Smith.

Table 2-1| Information of plasmids that were available for this thesis

Name	Description	Reference
pSC11GB	This plasmid expresses a green fluorescent protein/blasticidin resistant (GFP/bsd) fusion protein under the control of the VACV strong synthetic promoter from the *Hind*III site of plasmid pSC11-SBAKN (Chakrabarti et al., 1985). With this plasmid, transgenes can be inserted into the thymidine kinase (TK) gene in the VACV genome.	Wong et al. (2013)
pSC11GB-ES	It contains a GFP/bsd fusion gene under the control of the VACV strong synthetic promoter in the *Hind*III site of plasmid pSC11-ES (Eisenlohr et al., 1992a). This plasmid contained a multiple cloning site at the 3' end of the DNA sequencing encoding the ER-targeted signal peptide from the adenovirus E3/19K protein (amino acid sequence MRYMILGLLAL-AAVCSA). With this plasmid, transgenes carrying an ER-targeted sequence can be inserted into the TK gene in the VACV genome.	H. Korres and D.C. Tscharke (Unpublished)
P7.5GB-ins	This plasmid allows gene insertion into the intergenic region between the A11R and A12L genes in the VACV genome using a transient dominant selection with the GFP/bsd selection marker.	Wong et al. (2011)
pSSmCB	A modification version of plasmid p7.5GB (Wong et al., 2011). It contains a DNA cassette encoding a mCherry fluorescent protein-blasticidin resistant (mCherry/bsd) fusion protein under the control of the VACV strong synthetic promoter.	E. Spierings, S.A. Smith and D.C. Tscharke (Unpublished)

Table 2-1 (Continued)| Information of plasmids that were available for this thesis

Name	Description	Reference
pSC11GB-FullgB	A DNA fragment encoding the full-length HSV-1 glycoprotein B (gB) antigen was inserted into the plasmid pSC11GB	H. Korres and D.C. Tscharke (Unpublished)
pUC57-GIP	A plasmid that contains a DNA fragment encoding the eGFP-STS recombinant antigen, which consists of enhanced GFP (eGFP), OVA-257, B8-20 and gB-498 peptides. It was synthetised by GenScript.	T. Stefanovic and D.C. Tscharke (Unpublished)

Table 2-2| Information of plasmids that were generated in this thesis

Name	Description
pSC11GB-ESminigB	This plasmid contains an ER-targeted HSV gB-498 minigene under the control of the VACV p7.5 promoter. This plasmid was used to generate the recombinant virus MVA-ESmini-gB.
pSC11GB-FullPB1F2	This plasmid carries a full-length IAV basic polymerase 1 frame shift 2 (PB1F2) antigen under the control of the VACV p7.5 promoter. This plasmid was used to generate MVA-Full-PB1F2.
pSC11GB-ESminiPB1F2	This plasmid contains an ER-targeted IAV PB1F2-62 minigene under the control of the VACV p7.5 promoter. This plasmid was used to generate the recombinant virus MVA-ESmini-PB1F2.
pSC11GB-FullOVA	A DNA fragment encoding the full-length OVA antigen was inserted into the plasmid pSC11GB. This plasmid was used to generate the recombinant virus MVA-TK-OVA.
pSC11GB-mini OVA	This plasmid contains a cytosolic OVA-257 minigene under the control of the VACV p7.5 promoter. This plasmid was used to generate the recombinant virus MVA-TK-SIIN.
P7.5GB-insp7.5OVA	A DNA cassette encoding the full-length OVA antigen under the control of a VACV p7.5 promoter was cloned into plasmid p7.5GB_ins. This plasmid was used to generate the recombinant virus MVA-A11/A12-OVA.

Table 2-2 (Continued)| Information of plasmids that are generated in this thesis

Name	Description
P7.5GB-insp7.5miniOVA	A DNA cassette encoding the cytosolic OVA-257 minigene under the control of the VACV p7.5 promoter was cloned into the plasmid p7.5GB_ins. This plasmid was used to generate the recombinant virus MVA-A11/A12-SIIN.
pSSmCB-WR-A3	This plasmid contains a DNA fragment spanning the 5' upstream of A3L gene to part of the A3L encoding sequence from the WR genome.
pSSmCB-WR-EGFP-STS-A3	A DNA fragment encoding the eGFP-STS recombinant antigen, which was amplified from pUC57-GIP, was cloned into the plasmid pSSmCB-WR-A3 in a way that the inserted DNA sequence was placed directly after the start codon of A3L gene. This plasmid was used to generate the recombinant virus WR-eGFP-STS-A3.
pSSmCB-WR-eGFP-SKS-A4	A pSSmCB plasmid carrying a DNA sequence spanning the 5' upstream of A4L gene to part of the A4L encoding sequence from the WR genome. In addition, a sequence encoding a recombinant eGFP-SKS antigen, consisting of an eGFP, followed by the OVA-257, D3E-128 and gB-498 peptides, was placed directly after the start codon of A4L gene. This plasmid was used to generate the recombinant virus WR-eGFP-STS-A3.
pSSmCB-MVA-eGFP-SKS-A4	This plasmid is similar to that of plasmid pSSmCB-WR-eGFP-SKS-A4. However, the upstream sequence of A4L and the A4L encoding sequence was amplified from the MVA genome.

2.1.4 Oligonucleotides

All oligonucleotides used were synthesised by Sigma-Aldrich and are listed in Tables 2-3 and 2-4. The concentration of the working stock of each oligonucleotide used was 10 pmol/μl in sterile water.

Oligonucleotides shown in Table 2-3 were used to generate short double-stranded DNA inserts encoding peptide mingenes for cloning. This was done by annealing two complementary oligonucleotides together (Section 2.2.6). Oligonucleotides

shown in Table 2-4 were used as primers in PCR (Section 2.2.1) or DNA sequencing reactions (Section 2.2.11).

Synthetic phosphorothioated CpG1668 oligonucleotide, with the sequence TCCATGACGTTCCTGATGCT, was resuspended in 1 nmol/µl in sterile water. It was diluted to 0.1 nmol/µl in PBS before injection into mice (Section 2.2.23).

Table 2-3| Oligonucleotides used for the generation of short double-stranded DNA inserts

Name	Sequence	Encoded minigene
pSC11GB-miniOVAfor	GTGCACCATGTCTATAATAAACTTT GAGAAGTTATAGTGA	OVA-257 minigene
pSC11GB-miniOVArev	GGCCTCACTATAACTTCTCAAAGTT TATTATAGACATGGTGCACGGCC	OVA-257 minigene (complementary to pSC11GB-miniOVAfor)
pSC11ESGB-ESminiPB1F2for	GGCCCTTTCCTTGAGGAATCCCATC CTGGTATGATAGGATATC	PB1F2-62 minigene
pSC11ESGB-ESminiPB1F2rev	GGCCGATATCCTATCATACCAGGA TGGGATTCCTCAAGGAAAG	PB1F2-62 minigene (complementary to pSC11ESGB-ESminiPB1F2rev)
MiniGeneNotI/NotIF pSCIIES	GGCCTCCTCCATCGAGTTCGCCCG GCTGTGATAGGATATC	HSV gB-498 minigene
pSCGB-ESHSVgB498-bot	GGCCGATATCCTATCACAGCCGGG CGAACTCGATGGAGGA	HSV gB-498 minigene (complementary to pSC11ESGB-ESminiPB1F2rev)

Table 2-4| Oligonucleotides used as primers for PCR or DNA sequencing reactions

Name	Sequence	Use
pSC11GB-fullPB1F2for	GTACAGATCTGTACGG TGCACCATGGGACAG GAAC	To amplify full-length PB1F2 gene from the recombinant virus WR-Full-PB1F2
pSC11GB-fullPB1F2rev	TAGTTAGCTGCGGCCT CACTACTCGTGTTTGC TG	To amplify full-length PB1F2 gene from the recombinant virus WR-Full-PB1F2
pSC11_FullOVA for	GTACAGATCTGTACGC ACCATGGGCTCCAT	To amplify the DNA sequence encoding full-length OVA antigen from MVA-delIII-OVA
pSC11_FullOVA rev	TAGTTAGCTGCGGCCT TTCTAGATTAAGCT	To amplify the DNA sequence encoding full-length OVA antigen from MVA-delIII-OVA
infus_for_pSC11	TGCAGATATCACTAGC GTAGCATCGCTTCT	To amplify fragments encoding full-length OVA and OVA-257 miningene OVA from plasmids pSC11GB-FullOVA and pSC11GB-miniOVA respectively
infus_rev_pSC11	GAATGTATTAACTAGC CCTATAGTGTCACC	To amplify fragments encoding full-length OVA and OVA-257 miningene OVA from plasmids pSC11GB-FullOVA and pSC11GB-miniOVA respectively
infusion_A3L_for	CAGTGTGCTGGAATTT CCTATTGTGGCCAC	To amplify part of the A3L gene sequence from the VACV genome
infusion_A3L_328 _rev	GATATCTGCAGAATTC AATTGCATTCCCAT	To amplify part of the A3L gene sequence from the VACV genome
before_A3L_start	TATTTATATTCGTAGT TTTTACTCG	To amplify the whole plasmid pSSmCB-A3-328

Table 2-4 (Continued)| Oligonucleotides used as primers for PCR and DNA sequencing reactions

Name	Sequence	Use
after_A3L_start	GAAGCCGTGGTCAATAGC	To amplify the whole plasmid pSSmCB-A3-328
infus_for_GFP-STS	CTACGAATATAAATAATGGTGAGCAAGGGC	To amplify the sequence encoding the eGFP-STS recombinant antigen from plasmid pUC57-GIP
infus_rev_GFP-STS	ATTGACCACGGCTTCGGCCTTCAGCCGGGCGAACTC	To amplify the sequence encoding the eGFP-STS recombinant antigen from the plasmid pUC57-GIP
A4Lup_fwd	CAGTGTGCTGGAATTCTCGTTGAAGCAAGTTATAG	To amplify the upstream region of the A4L gene from the VACV genome
A4Lup_rev	TTAAGGCTTTAAAATTGAATTGC	To amplify the upstream region of the A4L gene from theVACV genome
A4L_fwd	AGTTCGCCCGGCTGAAGGCCGACTTCTTTAACAAGTTCTCAC	To amplify part of the A4L gene sequence from the VACV genome
A4L_rev	GATATCTGCAGAATTGACATATCAGATGATGTATTAG	To amplify part of the A4L gene sequence from the VACV genome
A4Lup_eGFP_fwd	GCAATTCAATTTTAAAGCCTTAAATGGTGAGCAAGGGC	Primer to amplify part of the DNA sequence encoding the eGFP-STS antigen for the generation of a DNA fragment encoding a new eGFP-SKS antigen
eGFP-SKS_rev	GGCCTTCAGCCGGGCGAACTCGATGGAGCTGGCCTTGAGGTTCTCATATTGCACCACTTTGGCCTTCAGTTTTTCAAAG	Primer to amplify part of the DNA sequence encoding the eGFP-STS antigen for the generation of a DNA fragment encoding a new eGFP-SKS antigen

Table 2-4 (Continued)| Oligonucleotides used as primers for PCR and DNA sequencing reactions

Name	Sequence	Use
VACV092fwd	GGATGACAACTCAAA CATCTGC	Diagnostic PCR primer for VACV recombinants with inserts in the VACV TK gene
laczpSC11rev	AGGCACATGGCTGAAT ATCG	Diagnostic PCR primer for VACV recombinants with inserts in the VACV TK gene
SP6	GATTTAGGTGACACTA TAG	Diagnostic PCR primer for VACV recombinants with inserts in the VACV TK gene
TKLrev1	GTCAGTCTCATGTTCT CACCG	Diagnostic PCR primer for VACV recombinants with inserts in the VACV TK gene
FwdHSV-gBend	ACACAAGGCCAAGAA GAAGG	Diagnostic PCR primer for the recombinant virus MVA-Full-gB
SeqGFPbsd600For	CAACTAGCAGACCATT ATC	Diagnostic PCR primer for VACV recombinants with inserts in the VACV TK gene
revGFPbsdend	CCTCCCACACATAACC AGA	Diagnostic PCR primer for VACV recombinants with inserts in the VACV TK gene
VACV095rev	ACGGACGATCTTATTA AGGTA	Diagnostic PCR primer for VACV recombinants with inserts in the VACV TK gene
WR130fwd	GGTAAGACTCCGTATA AATCAA	Diagnostic PCR primer for VACV recombinants with inserts in the A11R/A12L intergenic region

Table 2-4 (Continued)| Oligonucleotides used as primers for PCR and DNA sequencing reactions

Name	Sequence	Use
SeqGbF11	CGTCACTGTTCTTTAT GATTCTA	Diagnostic PCR primer for VACV recombinants with inserts in the A11R/A12L intergenic region
WR131rev	CTAGCGCAAATCGGTG GA	Diagnostic PCR primer for VACV recombinants with inserts in the A11R/A12L intergenic region
before_A3L_seq	GATCAAAAGACGACG ACACC	Diagnostic PCR primer for the recombinant virus WR-eGFP-STS-A3
rev_eGFP_seq	CTCGACCAGGATGGGC AC	Diagnostic PCR primer for the recombinant virus WR-eGFP-STS-A3
for_eGFP_524seq	CCACAACATCGAGGA CGG	Diagnostic PCR primer for the recombinant virus WR-eGFP-STS-A3
rev_A3_454seq	CATGGGTAAGGAGTTG AAATAG	Diagnostic PCR primer for the recombinant virus WR-eGFP-STS-A3
for_eGFP_524seq	CCACAACATCGAGGA CGG	Diagnostic PCR primer for recombinant viruses WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4
revWR123seq	ACGTCTGAGAAGGTTG GG	a) Diagnostic PCR primer for recombinant viruses WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4 b) Sequencing primer for the A4L gene
SeqRevpEGFPN1	CTCGACCAGGATGGGC AC	Diagnostic PCR primer for recombinant viruses WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4

Table 2-4 (Continued)| Oligonucleotides used as primers for PCR and DNA sequencing reactions

Name	Sequence	Use
fwdWR124seq	CTTTGTCAAAATCGTT TACCGAC	Sequencing primer for the A4L gene
SeqpSC11p7.5	CACTAATTCCAAACCC ACCC	Sequencing primer for inserts in the transfer plasmids pSC11GB and pSC11GB-ES and in the VACV TK gene from the VACV genome
SeqpSC11TK-R	TGAAATGTCCCATCGA GTGC	Sequencing primer for inserts in the transfer plasmids pSC11GB and pSC11GB-ES and in the VACV TK gene from the VACV genome
pSC11lacZseq	GTGCTGCAAGGCGATT AAGT	Sequencing primer for the inserts in the VACV TK gene from the VACV genome
Sequence SP6 rev	CTATAGTGTCACCTAA ATC	Sequencing primers for inserts in the transfer plasmids psC11GB and pSC11GB-ES
HSVgB p2(235)	CGCTACTCCCAGTTTA TGGGGAT	Sequencing primer for the full-length HSV gB antigen
HSVgB p4(237)	GAGTACGGCGGCTCCT TCCGAT	Sequencing primer for the full-length HSV gB antigen
HSVgB p6(239)	CGAGCTACAGAATCAC GAGCTGAC	Sequencing primer for the full-length HSV gB antigen
OvaSeqFor1	TCCTTCAGCCAAGCTC CGTG	Sequencing primer for the full-length OVA gene
Frd_seq_PCR_blu ntII_MCS	TACGCCAAGCTATTTA GGTG	Sequencing primer for inserts in plasmids p7.5GB, p7.5GB-ins, and pSSmCB

Table 2-4 (Continued)| Oligonucleotides used as primers for PCR and DNA sequencing reactions

Name	Sequence	Use
Rev_seq_PCR_ bluntII_MCS	CGATTAAGTTGGGTAA CGCC	Sequencing primer for inserts in p7.5GB, p7.5GB-ins, and pSSmCB
WR130-Fi	TATGCATCAAGCTTGC ATTATCGAGCAAGCAT TGC	Sequencing primer for inserts in A11R/A12L intergenic region from VACV genome
WR131-Fi	GCCAGTGTGATGGATT AGAGACGGCCAATTA GAAG	Sequencing primer for inserts in A11R/A12L intergenic region from VACV genome

2.1.5 Mice

Female specific pathogen-free C57Bl/6, B6.SJL, and F5 TCR transgenic mice (Mamalaki et al., 1992; Mamalaki et al., 1993) were obtained from the Animal Resources Centre (Perth, Australia) or the Australian Phenomics Facility (Canberra, Australia). Mice were housed and used for experiments at 7-14 weeks old. All experiments were done according to the relevant ethical requirements and were approved by the Australian National University Animal Ethics and Experimentation Committee (protocol numbers: F.BMB.38.08, A2011.001 and A2011.038). C57Bl/6 mice were used unless otherwise stated.

2.1.6 Cell lines

Cell lines used are listed in Table 2-5. They were maintained in D10 media at 37°C with 5% CO_2 in culture flasks and were subcultured twice a week. Cells were maintained as described in Section 2.2.12.

Table 2-5| Cell lines used in this thesis

Cell line	Origin	Use
293A [1]	Primary human embryonic kidney cells (transformed with human adenovirus 5 DNA)	a) For generating recombinant viruses with VACV strain WR background b) As antigen donor cells for in vivo cross priming experiments
293-D^b [2]	293A cells that stably express H-$2D^b$ under the control of the strong cytomegalovirus promoter	a) As stimulators used in the in vitro presentation experiments
293-K^b [2]	293A cells that stably express H-$2K^b$ under the control of the strong cytomegalovirus promoter	a) As stimulators used in the in vitro presentation assays b) As APCs for measuring OVA-257/H-$2K^b$ presentation after infection with VACVs expressing OVA constructs
BHK-21 [1]	Syrian golden Hamster kidney fibroblast	a) For MVA titration b) For generation and plaque purification of recombinant VACVs with MVA background c) For growing VACV stocks
BSC-1 [1]	African green monkey kidney epithelial cells	a) For VACV strain WR titration b) For plaque purification of recombinant VACVs with WR background
DC2.4 [3]	DC-like cell line generated from C57Bl/6 mouse. It expresses H-$2K^b$ and H-$2D^b$	a) As stimulators used in the in vitro direct presentation assays b) As APCs for measuring OVA-257/H-$2K^b$ presentation after infection with VACVs expressing OVA constructs

1 Refer to the American Type Culture Collection (ATCC; www.atcc.org), USA.
2 Tscharke et al. (2005).
3 Shen et al. (1997).

2.1.7 Synthetic Peptides

Synthetic peptides with the minimal amino acid sequences of $CD4^+$ or $CD8^+$ T cell epitopes were used for ex vivo stimulation of splenic $CD8^+$ T cells isolated from immunised mice (Section 2.2.29), peptide loading of DimerX (Section 2.2.31), or pulsing bone marrow-derived DCs (BMDCs) in vitro (2.2.34). The peptides were synthesised by GenScript unless otherwise stated. The peptides

used are listed in Table 2-6. Master stock of peptides were prepared at a concentration of 1 or 10 mg/ml in DMSO and stored in a -80°C freezer. Peptides were diluted with D0 media or PBS before use.

Table 2-6| Synthetic peptides used in this thesis

Peptide name [1]	Amino acid sequence	Origin of peptide [2]	MHC-I Restriction	Reference
A42-88	YAPVSPIVI	VACV, A42, 88-96	H-2D^b	Tscharke et al. (2005)
A8-70	IHYLFRCV	VACV, A8, 70-77	H-2K^b	Moutaftsi et al. (2006)
J3-289	SIFRFLNI	VACV, J3, 289-296	H-2K^b	Moutaftsi et al. (2006)
C4-125	LNFRFENV	VACV, C4, 125-132	H-2K^b	Moutaftsi et al. (2006)
A3-191	YSPSNHHIL	VACV, A3, 191-199	H-2K^b	Moutaftsi et al. (2006)
A47-171 [3]	YAHINALEYI	VACV, A47, 171-180	H-2K^b	Yuen et al. (2010)
L2-53	VIYIFTVRL	VACV, L2, 53-61	H-2K^b	Moutaftsi et al. (2006)
A47-138	AAFEFINSL	VACV, A47, 138-146	H-2K^b	Tscharke et al. (2005)
B2-54	YSQVNKRYI	VACV, B2, 54-62	H-2D^b	Moutaftsi et al. (2006)
K3-6	YSLPNAGDVI	VACV, K3, 6-15	H-2D^b	Tscharke et al. (2005)
A23-297	IGMFNLTFI	VACV, A23, 297-305	H-2D^b	Moutaftsi et al. (2006)
A3-270	KSYNYMLL	VACV, A3, 270-277	H-2K^b	Moutaftsi et al. (2006)
A8-189	ITYRFYLI	VACV, A8, 189-196	H-2K^b	Moutaftsi et al. (2006)
B8-20	TSYKFESV	VACV, B8, 20-27	H-2K^b	Tscharke et al. (2005)
OVA-257	SIINFEKL	Chicken, OVA, 257-264	H-2K^b	Falk et al. (1991b) and Rötzschke et al. (1991)

Table 2-6 (Continued)| Synthetic peptides used in this thesis

Peptide name [1]	Amino acid sequence	Origin of peptide [2]	MHC-I Restriction	Reference
gB-498	SSIEFARL	HSV-1, glycoprotein B (gB), 498-505	H-2K^b	Bonneau et al. (1993)
PB1-703 [3]	SSYRRPVGI	IAV strain A/Puerto Rico/8/1934 (PR8), basic polymerase 1 (PB1), 703-711	H-2K^b	Belz et al. (2001)
PB1F2-62	LSLRNPILV	IAV strain PR8, PB1 frame shift 2 (PB1F2), 62–70	H-2D^b	Chen et al. (2001a)
PA-224	SSLENFRAYV	IAV strain PR8, acid polymerase (PA), 224-233	H-2D^b	Belz et al. (2000)
NS2-114	RTFSFQLI	IAV strain PR8, non-structural 2 (NS2), 114-121	H-2K^b	Vitiello et al. (1996)
PR8NP-366	ASNENMETM	IAV strain PR8, nucleoprotein (PR8NP), 366-374	H-2D^b	Rotzschke et al. (1990)
NT60NP-366	ASNENMDAM	IAV strain A/Northern Territory/60/1968 (NT60), nucleoprotein (NT60NP), 366-374	H-2D^b	Rotzschke et al. (1990)
PR8NP-366-pS	Ap(S)NENMETM	Same sequence as PR8NP366, with serine at position 2 phosphorylated	H-2D^b (Assumed)	-
D3E-128	KVVQYENL	Dengue virus type 3, envelope protein, 128-135	H-2D^b (Predicted)	B. Quinan and D.C. Tscharke (Unpublished)
E1A-324	SGPSNTPPEI	Adenovirus type 5, early 1A protein, 234-243	H-2D^b	Kast and Melief (1991)
HBcAg-128	TPPAYRPPNAPIL	Hepatitis B virus, core antigen, 128-140	H-2I-A^b	Milich et al. (1988) and Alexander et al. (1994)

1 The name for each peptide is based on the name of the protein that contains the particular sequence.

2 The origin, the name of the protein that contained the peptide sequence, followed by the residue number of the peptide within the protein is shown for each peptide.

3 The A47-171 peptide was synthesised by Mimotopes. The PB1-703 peptide was provided by the laboratory of Professor S. Turner (University of Melbourne, Australia).

2.1.8 Viruses

Viruses used in this study that were already available are listed in Table 2.7. The majority of these viruses were obtained from Dr. J.R. Bennink and Dr. J.W. Yewdell (National Institute of Allergy and Infectious Diseases, USA), unless otherwise stated. VACV recombinants generated in this thesis are listed in Table 2.8. Some viruses were generated with the assistance of S.A. Smith. It should be noted that for most recombinant viruses which express transgenes from the TK locus, a *lacZ* gene under the control of the late VACV p11 promoter was also inserted into the TK locus, except for WR-Full-PA and WR-Full-NS2. In addition, the ER-targeted sequence used in some recombinant viruses is the ER-targeted signal peptide from the adenovirus E3/19K protein (Eisenlohr et al., 1992a), unless otherwise stated. Please also note that tables showing the viruses used in each chapter are reproduced in the Appendix for the ease of reference.

Table 2-7| Viruses that were already available for this thesis

Virus	Description	Reference
WR [1]	Wildtype VACV strain WR	Parker et al. (1941) and Bronson and Parker (1941)
WR-delTK	WR with the TK locus knocked out	Chakrabarti et al. (1985)
WR-TK-OVA	WR expressing full-length OVA by the VACV p7.5 promoter from the TK locus	Restifo et al. (1995)
WR-TK-SIIN	WR expressing a cytosolic OVA-257 minigene by the VACV p7.5 promoter from the TK locus	Bacik et al. (1994)
WR-Full-gB [2]	WR expressing full-length HSV gB antigen by the p7.5 promoter from the TK locus	Lin et al. (2013)
WR-ESmini-gB [3]	WR expressing an ER-targeted gB-498 minigene by the p7.5 promoter from the TK locus	Blaney et al. (1998)
WR-Full-PB1	WR expressing full-length PB1 from IAV strain PR8 by the p7.5 promoter from the TK locus	Chen et al. (2001a)

Table 2-7 (Continued)| Viruses that were already available for this thesis

Virus	Description	Reference
WR-ESmini-PB1	WR expressing an ER-targeted PB1-703 minigene by the p7.5 promoter from the TK locus	Unpublished
WR-Full-PB1F2	WR expressing full-length PB1F2 from IAV strain PR8 by the p7.5 promoter from the TK locus	Chen et al. (2001a)
WR-ESmini-PB1F2	WR expressing an ER-targeted PB1F2-62 minigene by the p7.5 promoter from the TK locus	Lev et al. (2009)
WR-Full-PA	WR expressing a full-length PA from IAV strain PR8 by the p7.5 promoter from the TK locus	Smith et al. (1987)
WR-ESmini-PA	WR expressing an ER-targeted PA-224 minigene by the p7.5 promoter from the TK locus	Lev et al. (2009)
WR-Full-NS2	WR expressing a full-length NS2 antigen from IAV strain A/Udorn/1972 by the p7.5 promoter from the TK locus	Smith et al. (1987)
WR-ESmini-NS2	WR expressing an ER-targeted NS2-114 minigene by the p7.5 promoter from the TK locus	Unpublished
WR-Full-PR8NP	WR expressing a full-length PR8NP antigen by the p7.5 promoter from the TK locus	Restifo et al. (1995)
WR-ESmini-PR8NP	WR expressing an ER-targeted PR8NP-366 minigene by the p7.5 promoter from the TK locus	Lev et al. (2009)
WR-mini-PR8NP	WR expressing a cytosolic PR8NP-366 minigene by the p7.5 promoter from the TK locus	Lev et al. (2009)
WR-Full-T60NP	WR expressing a full-length NT60NP antigen by the p7.5 promoter from the TK locus	Townsend et al. (1988)
WR-ESmini-NT60NP	WR expressing an ER-targeted NT60NP-366 minigene by the p7.5 promoter from the TK locus. The ER-targeted sequence was the signal sequence of the hemagglutinin protein from IAV strain PR8.	Elliott et al. (1995)

Table 2-7 (Continued)| Viruses that were already available for this thesis

Virus	Description	Reference
WR-NP-S-GFP	WR expressing a recombinant NP-S-GFP antigen consisting of PR8NP, OVA-257 peptide and eGFP by the p7.5 promoter from the TK locus	Antón et al. (1999)
WR-UbR-NP-S-GFP	WR expressing the NP-S-GFP antigen tagged with an ubiquitin protein and an arginine residue at the amino-terminus. The transgene was driven by the p7.5 promoter from the TK locus.	Princiotta et al. (2003)
WR-cyto-PR8NP	WR expressing the amino acid residues of 13-498 of PR8NP under the control of p7.5 promoter from the TK locus	Golovina et al. (2002)
WR-ES-PR8NP	WR expressing a recombinant antigen consisting of the amino acid residues of 13-498 of PR8NP tagged with an ER-targeted sequence under the control of p7.5 promoter from the TK locus. The ER-targeted sequence was the leader sequence of the hemagglutinin protein from IAV strain A/WSN/33.	Golovina et al. (2002)
WR-delB8 [4]	WR with B8R deleted	Symons et al. (2002)
WR-delB8-miniB8 [2]	WR with the B8R gene replaced by a cytosolic B8-20 minigene	Wong et al. (2011)
MVA	Wildtype VACV strain MVA	Mayr et al. (1975)
MVA-delIII-OVA [5]	MVA expressing full-length OVA by the VACV p7.5 promoter from the deletion III (delIII) region	Gasteiger et al. (2007)
MVA-delIII-SIIN [5]	MVA expressing a cytosolic OVA-257 minigene by the VACV p7.5 promoter from the delIII region	Gasteiger et al. (2007)
MVA-delB8 [5]	MVA with B8R deleted	Kastenmuller et al. (2007)
MVA-delB8-miniB8 [2]	MVA with the B8R gene replaced by a cytosolic B8-20 minigene	Y.C. Wong and D.C. Tscharke (unpublished)

Table 2-7 (Continued)| Viruses that were already available for this thesis

Virus	Description	Reference
IAV strain PR8 [6]	Wildtype IAV strain PR8	-
IAV-B8 [7]	IAV strain PR8 with B8-20 peptide inserted into the neuraminidase stalk	S.A. Smith, T. Cukalac and D.C. Tscharke (Unpublished)
HSV strain KOS [8]	Wildtype HSV strain KOS	Rawls et al. (1968)

1 The virus was provided by Dr. B. Moss (National Institute of Allergy and Infectious Diseases, U.S.A.).
2 The viruses were generated in the Tscharke lab (Australia National University, Australia).
3 The virus was provided by Professor S.S. Tevethia (Pennsylvania State University, U.S.A.)
4 The virus was provided by Professor G.L. Smith (University of Cambridge, UK).
5 They were provided by Professor I. Drexler (Heinrich-Heine-Universität, Germany)
6 The virus was obtained from the laboratory of Professor C. Goodnow (Australia Nation University, Australia).
7 The virus was generated in the laboratory of Professor S. Turner (University of Melbourne, Australia).
8 The virus was provided by Professor F. Carbone (University of Melbourne, Australia).

Table 2-8| VACV recombinants generated in this thesis

Virus	Comment
MVA-delTK	MVA with the TK locus knocked out
MVA-Full-gB	MVA expressing full-length gB antigen by the p7.5 promoter from the TK locus
MVA-ESmini-gB	MVA expressing an ER-targeted gB-498 minigene by the p7.5 promoter from the TK locus
MVA-Full-PB1F2	MVA expressing a full-length PB1F2 antigen by the p7.5 promoter from the TK locus
MVA-ESmini-PB1F2	MVA expressing an ER-targeted PB1F2-62 minigene by the p7.5 promoter from the TK locus
MVA-TK-OVA	MVA expressing full-length OVA by the VACV p7.5 promoter from the TK locus

Table 2-8 (Continued) | VACV recombinants generated in this thesis

Virus	Comment
MVA-TK-SIIN	MVA expressing a cytosolic OVA-257 minigene by the VACV p7.5 promoter from the TK locus
MVA-A11/A12-OVA	MVA expressing full-length OVA by the VACV p7.5 promoter from the VACV A11R/A12L intergenic region
MVA-A11/A12-SIIN	MVA expressing a cytosolic OVA-257 minigene by the VACV p7.5 promoter from the VACV A11R/A12L intergenic region
MVA-delIII-OVA-delTK	MVA expressing full-length OVA by the VACV p7.5 promoter from the delIII region, with TK gene knockout
MVA-delIII-SIIN-delTK	MVA expressing a cytosolic OVA-257 minigene VACV p7.5 promoter from the delIII region, with TK gene knockout
WR-eGFP-STS-A3	WR with A3 antigen tagged with a recombinant antigen consisting of eGFP, OVA-257, B8-20 and gB-498 peptides (eGFP-STS recombinant antigen) at the amino-terminus.
WR-eGFP-SKS-A4	WR with A4 antigen tagged with a recombinant antigen consisting of eGFP, OVA-257, D3E-128, and gB-498 peptides (eGFP-SKS recombinant antigen) at the amino-terminus
MVA-eGFP-SKS-A4	MVA with A4 antigen tagged with the eGFP-SKS recombinant antigen at the amino-terminus

2.1.9 Antibodies and anti-VACV hyperimmune serum

Commercially available antibodies used in this thesis are listed in Table 2.7. Most of the antibodies were used to label cells for flow cytometric analysis. Anti-rabbit-IgG-peroxidase was used as the secondary antibody for immunostaining of MVA-infected cell monolayers during titration of MVA (Section 2.2.17.2).

In addition to the commercially available antibodies, rabbit anti-VACV hyperimmune serum was used as the primary antibody for immunostaining of MVA-infected cell monolayers for MVA titration (Section 2.2.17.2). It was generated by four immunisations of a rabbit at three weekly intervals with VACV

strain MVA (1×10^7 PFU) and serum was collected two weeks after the last immunisation (Institute of Medical and Veterinary Science, Australia).

When indicated, culture supernatant from the 2.4G2 hybridoma, which expresses anti-mouse-CD16/CD32 antibody, was use to block receptors specific for the fragment crystallisable region (Fc) of antibody expressed on cells before further antibody labelling.

Table 2-9| Commercially available antibodies used in this thesis

Antibody (-conjugate)	Species	Clone	Source
Anti-mouse IFN-γ-APC [1]	Rat	XMG1.2	Biolegend
Anti-mouse-CD8α-PE [2]	Rat	53-6.7	Biolegend
Anti-mouse-CD8α-PE-Cy7 [3]	Rat	53-6.7	Biolegend
anti-OVA-257/H-2K^b-APC	mouse	25D1.16	Biolegend / eBioscience
Anti-mouse-TNF-α-PE-Cy7	rat	MP6-XT22	BD Pharmingen
Anti-mouse-IL-2-α-PE-Cy5 [4]	rat	JES6-5H4	Biolegend
Anti-mouse-CD45.2-APC	mouse	104	Biolegend
Anti-mouse-CD69-PE-Cy7	Armenian Hamster	H1.2F3	Biolegend
Anti-mouse-CD25-PE	Rat	PC61	Biolegend
Anti-mouse-CD11c-APC	Armenian Hamster	N418	Biolegend
Anti-mouse-IgG1-PE	Rat	A85-1	BD Pharmingen
Anti-rabbit-IgG-peroxidase [5]	Goat	Polyclonal	Sigma-Aldrich

1 APC: allophycocyanin
2 PE: phycoerythrin
3 PE-Cy7: phycoerythrin-cyanine 7
4 PE-Cy5: phycoerythrin-cyanine 5
5 The antibody is conjugated to horseradish peroxidase

2.2 Methods

2.2.1 Polymerase chain reaction (PCR)

PCR was performed with either Phusion DNA polymerase (New England Biolabs) or *Taq* DNA Polymerase (New England Biolabs).

Phusion DNA polymerase has high fidelity to reduce the rate of errors in the PCR product sequence. This enzyme was used wherever the product was to be incorporated into a recombinant virus. In a PCR with Phusion DNA polymerase, 0.2 ml tubes were set up with a final reaction volume of 50 µl in sterile water, including 1× Phusion HF Buffer, 200 µM dNTPs, 0.5 µM of each forward and reverse primer, 2 µl DNA template (either purified plasmids (Section 2.2.9) or viral genomic DNA (Section 2.2.15)) and 1 unit of Phusion DNA polymerase. The samples were then run in a thermocycler (a Veriti 96-well Thermal Cycler (Life Technologies) or a Mastercycler (Eppendorf)). Sample thermal cycling conditions are shown below:

a. 98°C for 30 seconds (sec) (Initial denaturation)
b. 30 cycles of:
   - 98°C for 10 sec (Denaturation)
   - 45-72°C for 30 sec (Annealing temperature was determined from the primers used)
   - 72°C for 15 sec/1,000bp (Extension)
c. 72°C for 10 minutes (min) (Final extension)
d. Hold at 4°C.

Taq DNA Polymerase has lower fidelity and was used for the diagnostic PCRs to screen recombinant viruses (Section 2.2.14). It was also used to produce DNA fragments from the recombinant viruses for DNA sequencing (Section 2.2.11). In a PCR with *Taq* DNA polymerase, 0.2 ml tubes were set up with a final reaction volume of 25 µl in sterile water, including 1× ThermoPol reaction buffer, 200 µM of dNTPs, 0.35 µM of each forward and reverse primer, 1 µl of prepared viral genomic DNA from isolated plaques (Section 2.2.15) and 0.5 unit *Taq* DNA

polymerase. The samples were then run in a thermocycler as above. Sample thermal cycling conditions are shown below:

a. 95°C for 30 sec (Initial denaturation)
b. 30 cycles of:
   - 95°C for 30 sec (Denaturation)
   - 45-65°C for 30 sec (Annealing temperature was determined from the primer pairs used)
   - 68°C for 1 min/kb (Extension)
c. 68°C for 10 min (Final extension)
d. Hold at 4°C

2.2.2 Restriction enzyme digestion

Purified plasmids were digested with different restriction endonucleases. This was to either produce linearised plasmids for cloning (Section 2.2.7) or for restriction digestion analysis of recombinant plasmids cloned with DNA inserts (Section 2.2.9). In general, each restriction digestion reaction (20-50 µl) contained 1× NEBuffer provided, 5-100 ng/µl purified plasmids, 0.1-0.4 unit/µl restriction enzymes in sterile water. When required, 1× bovine serum albumin solution was added. The reactions were then incubated at 37°C for 2-4 hours (h). When the *Apa*I restriction enzyme was used, the reaction was carried out at 25°C.

2.2.3 Agarose gel electrophoresis

To visualise the PCR products or restriction-digest fragments of plasmids, samples were run on a 1% agarose gel in 1X TAE by gel electrophoresis which separates DNA fragments according to their lengths. Horizontal electrophoresis apparatuses (Bio-Rad) were used. Firstly, a 1% agarose gel was casted. Samples were mixed with appropriate volumes of 6× DNA loading dye. 6-15 µl of the solution was loaded into wells of the agarose gel. DNA ladder was loaded into the gels and used as a molecular size and mass standard. Gel electrophoresis was then performed at 90 to 120V for 30 min to 1 h in an electrophoresis chamber. The gel was stained with DNA staining solution for at least 15 min, followed by de-

staining in water. The gel was visualised with a UV transilluminator system (Vilber Lourmat).

2.2.4 Determination of DNA concentration

The concentration of purified plasmids or PCR products was determined based on the absorbance of light at 260 nm wavelength using a NanoDrop UV/Vis spectrophotometer (Thermo Scientific).

2.2.5 Purification of PCR products and linearised plasmids obtained from restriction enzyme digestion

Two different approaches were used. The first approach involved purification of DNA samples directly from reaction solutions while the second approach required gel purification. In both cases, the Geneclean spin kit (MP Biomedicals) was used according to the manufacturer's instructions.

2.2.6 Annealing of single-stranded synthetic oligonucleotides

Oligonucleotides shown in Table 2-3 were annealed with their complementary oligonucleotides to generate short double-stranded DNA inserts encoding peptide minigenes for cloning into transfer plasmids (Section 2.2.7.1). Briefly, two complementary single-stranded oligonucleotides at concentrations of 10 pmol/µl were mixed in a molar ratio of 1:1. The annealing reaction was then performed in a thermocycler with the following reaction conditions:

a. 95°C for 5 min
b. 70 cycles of 1 min incubation starting at 95°C, with the incubation temperature decreased by 1°C per cycle, until a temperature of 25°C was reached.

The reaction mix was then stored at 4°C before use.

2.2.7 Cloning of DNA inserts into plasmids

Two different methods were used to insert DNA fragments into plasmids: ligation with T4 DNA ligase or In-Fusion cloning (Clontech).

2.2.7.1 DNA ligation

The LigaFast rapid DNA ligation system (Promega) was used to ligate restriction-linearised plasmid with double-stranded oligonucleotides (Section 2.2.6) that carried the same restriction-overhangs. DNA ligation was performed in different molar ratio of plasmids to inserts. In general, a 10 µl ligation reaction contained 0.01-0.02 pmol of linearised plasmid, 0.1-10 pmol of DNA inserts, 1× rapid ligation buffer, and 2 units of T4 DNA ligase. The reaction was incubated at room temperature for 15 min and was then stored on ice. The product was then used for transformation (Section 2.2.8). The plasmids shown in Table 2-10 were created with this method.

Table 2-10| Plasmids generated using the DNA ligation strategy

Plasmid produced	Parental plasmid	Restriction enzymes [1]	Annealed oligonucleotides	Antigen encoded by the Insert
pSC11GB-miniOVA	pSC11GB	*Apa*I and *Not*I	1. pSC11GB-miniOVAfor 2. pSC11GB-miniOVArev	Cytosolic OVA-257 minigene
pSC11GB-ESminigB	pSC11GB-ES	*Not*I	1. MiniGeneNotI/NotI FpSCIIES 2. pSCGB-ESHSVgB498-bot	ER-targeted gB-498 minigene
pSC11GB-ESminiPB1F2	pSC11GB-ES	*Not*I	1. pSC11ESGB-ESminiPB1F2for 2. pSC11ESGB-ESminiPB1F2rev	ER-targeted PB1F2-62 mingene

1 Restriction enzymes used to linearise parental plasmids

2.2.7.2 In-Fusion cloning strategy

In-Fusion cloning (Clontech) uses homologous recombination to insert PCR products into linearised plasmids. The PCR products used as inserts for In-Fusion cloning were amplified with primers containing 15 bp-long extensions at their 5' ends homologous to the sequences flanking the insertion sites of the plasmids of choice. This allowed homologous recombination catalysed by the In-Fusion

enzyme to occur between the ends of the digested plasmids and the ends of PCR products. In general, each 10 µl In-Fusion reaction contained 1× In-Fusion Reaction buffer, 1 µl In-Fusion enzyme, a PCR insert, a linearised plasmid (in a 2:1 molar ratio of insert : plasmid) and sterile water. The cloning reaction was performed by incubating the reaction at 37°C for 15 min, followed by 50°C for 15 min in a PCR machine. The cloning reaction mix was diluted with 40 µl TE buffer and was used for transformation (Section 2.2.8).

The plasmids listed in Table 2-11 were generated with this approach. The generation of plasmids pSSmCB-WR-eGFP-SKS-A4 and pSSmCB-MVA-eGFP-SKS-A4 are described separately.

Table 2-11| Plasmids generated using the In-Fusion Cloning strategy

Plasmid produced	Parental plasmid	Restriction enzymes [1]	Generation of the insert fragment		Insert information
			DNA template	primers	
pSC11GB-FullPB1F2	pSC11 GB	*Apa*I and *Not*I	Genomic DNA from virus WR-Full-PB1F2	1. pSC11GB-fullPB1F2for 2. pSC11GB-fullPB1F2rev	Full-length PB1F2
pSC11GB-FullOVA	pSC11 GB	*Apa*I and *Not*I	Genomic DNA from virus MVA-delIII-OVA	1. pSC11_Full OVAfor 2. pSC11_Full OVArev	Full-length OVA
pSSGB-insp7.5 OVA	p7.5GB_ ins	*Spe*I	Plasmid pSC11GB-FullOVA	1. infus_for_pSC 11 2. infus_rev_pSC 11	Full-length OVA
pSSGB-insp7.5 miniOVA	p7.5GB_ ins	*Spe*I	Plasmid pSC11GB-miniOVA	1. infus_for_pSC 11 2. infus_rev_pSC 11	Cytosolic OVA-257 minigene

Table 2-11 (Continued)| Plasmids generated using the In-Fusion Cloning strategy

Plasmid produced	Parental plasmid	Restriction enzymes [1]	Generation of the insert fragment		Insert information
			DNA template	primers	
pSSmCB-WR-A3	pSSmCB	*Eco*RI	Genomic DNA from VACV WR	1. infusion_A3L_for 2. infusion_A3L_328_rev	326bp upstream to the first 328bp of the A3L gene
pSSmCB-WR-eGFP-STS-A3	pSSmCB-WR-A3	- [2]	pUC57-GIP	1. infus_for_GFP-STS 2. infus_rev_GFP-STS	DNA fragment encoding eGFP-STS was inserted into the 5' end of the A3L gene

1 Restriction enzymes used to linearise parental plasmids

2 A linearised DNA fragment of plasmid pSSmCB-WR-A3 was created by PCR with primers before_A3L_start and after_A3L_start.

Plasmid pSSmCB-WR-eGFP-SKS-A4

A DNA sequence spanning from -323 bp to -1 bp upstream of the start of the WR A4L gene was amplified from the WR genome using primers A4Lup_fwd and A4Lup_rev (Fragment 1). A second DNA fragment corresponding to the sequence of +3 bp to +341 bp of the WR A4L gene was amplified with primers A4L_fwd and A4L_rev from the WR genome (fragment 2). A third DNA fragment, which encoded the eGFP-SKS recombinant antigen, was generated by PCR using primers A4Lup_eGFP_fwd and eGFP-SKS_rev, with plasmid pUC57-GIP as the DNA template (fragment 3). An overlap extension PCR was then performed with fragments 1, 2, and 3 as templates, together with primers A4Lup_fwd and A4L_rev. This created a PCR product containing the upstream region of the A4L gene, following by the DNA fragment encoding the eGFP-SKS antigen which was inserted directly after the start codon of A4L gene, and the DNA sequence spanning from +3 bp to +341 bp of the WR A4L gene. This fragment was cloned into the *Eco*RI-digested pSSmCB using In-Fusion cloning strategy to create the plasmid pSSmCB-WR-eGFP-SKS-A4.

Plasmid pSSmCB-MVA-eGFP-SKS-A4

Plasmid pSSmCB-MVA-eGFP-SKS-A4 was created using the same strategy as the plasmid pSSmCB-WR-eGFP-SKS-A4. Here the upstream fragment and the A4L fragment were amplified from MVA genomic DNA. The primer sets used were identical to those used for generation of pSSmCB-WR-eGFP-SKS-A4.

2.2.8 Transformation

For each transformation, 5 µl DNA ligation product or diluted In-Fusion reaction product was mixed with 50-100 µl of competent *E.coli* cells and incubated on ice for 30 min, followed by heat shock at 42°C for 30 sec and then incubated on ice for 2 min. The transformed cells were incubated at 37°C for 1 h in 900 µl SOC medium with shaking. 100 µl of the transformants was then plated on LB-agar plates with either 100 µg/ml ampicillin (Sigma-Aldrich) or 50 µg/ml kanamycin (Sigma-Aldrich), depending on the antibiotic resistance genes in the plasmids. The plates were then incubated at 37°C overnight. Plasmids from colonies were then purified (Section 2.2.9).

2.2.9 Plasmid purification

To purify plasmids from transformed bacterial cells, the AxyPrep plasmid miniprep kit (Axygen) was used. Colonies on LB-agar plates with transformed bacterial cells were picked and allowed to grow in LB media containing the appropriate antibiotic at 37°C overnight (100 µg/ml ampicillin or 50 µg/ml kanamycin). One day later, plasmids from the bacterial cells were isolated according to the manufacturer's instructions.

2.2.10 Colony cracking for plasmid screening

Colony cracking strategy was used occasionally as a method to screen a large number of bacterial colonies transformed with newly generated plasmids. Briefly, bacterial colonies grown on LB-agar plates were picked. Some of the material was transferred to a new LB-agar plate supplemented with the appropriate antibiotics while the remaining bacterial cells were transferred to new microcentrifuge tubes. Each sample was resuspended in 40 µl of 10 mM EDTA (pH 8). 50 µl of cracking buffer containing 10 mg sucrose, 0.5% sodium N-lauroylsarcosinate and 200 mM

sodium hydroxide was mixed with the resuspended bacteria. The solution was incubated at room temperature for 5 min. 10 µl marker solution containing 8 mM EDTA (pH 8), 0.48 mM potassium chloride, and 0.03% bromophenol blue was added. The solution was then incubated on ice for 5 min, followed by centrifugation at 14,000g for 3 min. The supernatant was then used as the sample for agarose gel electrophoresis (Section 2.2.3). Supercoiled DNA ladder was used as the molecular size standard.

2.2.11 DNA sequencing

DNA sequencing was used to check the sequence of the DNA inserts in plasmids or recombinant viruses. To sequence an insert from a recombinant virus, viral DNA was first extracted from a virus seed stock (Section 2.2.16.1) and the insert region was amplified by PCR with *Taq* DNA polymerase to produce a DNA template for sequencing.

Each DNA sequencing reaction mix contained 1 µl of BigDye terminator (Life Technologies), 3.2 pmol primer, 3.5 µl of 5× sequencing buffer and a DNA template (10-40 ng for PCR products or 150-300 ng for purfied plasmids) in a final volume of 20 µl in sterile water. The extension reaction was then performed in a PCR machine with the following reaction conditions:

a. 94°C for 5 min (Initial denaturation)
b. 30 cycles of:
   - 96°C for 10 sec (Denaturation)
   - 50°C for 5 sec (Annealing temperature)
   - 60°C for 4 min (Extension)
c. Hold at 4°C

Sequencing reaction products were purified according to the instructions provided by the Biomolecular Resource Facility (John Curtin School of Medical Research, the Australian National University). In brief, for each 20 µl sequencing reaction, the DNA product was precipitated by mixing with 80 µl of solution containing 75 mM sodium acetate (pH4.6), 3.125 mM EDTA (pH 8), 75% ethanol. The solution

was incubated at room temperature for 15 min, followed by centrifugation at 14,000 g for 20 min. The supernatant were removed and the 250 µl of 70% ethanol was added. The solution was centrifuged at 14,000 g for 5 min. The product was dried by air and sent to Biomolecular Resource Facility (John Curtin School of Medical Research, the Australian National University) for DNA sequencing. VectorNTI software (Life Technologies) was used to analysed the obtained results.

2.2.12 Maintenance and preparation of mammalian cell lines

To prepare mammalian cell lines from a culturing flask, media were removed, followed by washing with PBS. The cells were then treated with 0.05% trypsin (Life Technologies) in PBS at 37°C until cells detached. The cells were then diluted with D10 media accordingly. Cells were transferred into new culturing flasks (Nunc) or plated into wells in 6-well or 96-well flat-bottom tissue-culture plates (Corning). The flasks and plates were incubated at 37°C with 5% CO_2. The flasks and plates were used when the cells had grown into a confluent monolayer. In some experiments, a single cell suspension was prepared. Viable cells were counted after staining with trypan blue solution using a haemocytometer or a Countess automated cell counter (Life Technologies).

2.2.13 Generation of recombinant viruses

Homologous DNA recombination between the transfer plasmid that carried the gene of interest and the viral genome within VACV-infected cells was used to generate VACV recombinants. After a DNA sequence of interest was cloned into a transfer plasmid (Section 2.2.7), the plasmid was transfected into VACV-infected cells to generate VACV recombinants. Briefly, confluent 293A or BHK-21 cell monolayers in 6-well plates were infected with WR or MVA respectively at a multiplicity of infection (m.o.i.) of 0.05 in D2 media. The cells were incubated at 37°C with 5% CO_2 for 1 h. During this incubation, a transfection mix was prepared by mixing 5 ng/µl of plasmid in D0 with 1% (v/v) Lipofectamine 2000 (Life Technologies) in D0 in equal volume by gently mixing. The transfection mix was incubated at room temperature for 30 min. After 1 h of infection, the infectious inoculum on each cell monolayer was aspirated and

replaced with 600 µl of D0. Transfection mix in a volume of 400 µl was then added to each well drop-wise. The plate was incubated for 2 h at 37°C in 5% CO_2 for the transfection mix to be taken up by the VACV-infected cells. The transfection mix was then aspirated and 2 ml D2 was added. The cells were incubated for two days at 37°C in 5% CO_2. During the infection/transfection process, plasmids carrying sequences homologous to the insertion sites of choice in the viral genome were expected to incorporate into the insertion sites by homologous recombination. After two days of incubation, the infected/transfected cells were harvested. The cells were frozen at -80°C and thawed in a 37°C water bath three times to lyse the infected cells and release the viruses. The cell lysate was then sonicated for 20 sec three times. The cell lysate was expected to contain both wildtype and recombinant viruses. The samples were ready to be used for plaque purification (Section 2.2.14). When not used, the samples were stored at -80°C.

2.2.14 Isolation of recombinant viruses via transient dominant selection

After the generation of VACV recombinants (Section 2.2.13), they needed to be isolated from wildtype viruses via serial steps of plaque purification. BSC-1 and BHK-21 cells were used for plaque purification of recombinant viruses from VACV WR and MVA backgrounds, respectively. Transient dominant selection was employed here, such that intermediate recombinants were selected first, followed by screening of final recombinants (Falkner and Moss, 1990). Two related selection markers were used. The first one is the GFP/bsd fusion protein (Wong et al., 2011) and the other one is the mCherry/bsd fusion protein (E. Spierings, S.A. Smith and D.C. Tscharke, unpublished). Both markers allow selection of intermediate recombinant viruses with antibiotic blasticidin S. The expression of GFP or mCherry fluorescent protein allowed a visual screening for intermediate recombinant viruses with fluorescence microscopy (Olympus microscope CKX41, equipped with reflected fluorescence illuminator CKX-RFA).

Briefly, cell lysates from infection/transfection process (Section 2.2.13) were serially diluted in 5-fold steps with D2 and added to confluent monolayers of

BSC-1 (for WR) or BHK-21 (for MVA) cells in 6-well plates. After 2 h of incubation at 37°C with 5% CO_2, the inoculum in each well was replaced with phenol red-free CMC plaque media containing 15 µg/ml antibiotic blasticidin S. Phenol red-free media were used to avoid auto-fluorescence associated with the pH indicator. The infected plates were incubated for two days at 37°C with 5% CO_2. Blasticidin S kills cells that do not express the blasticidin S resistance gene by inhibiting protein synthesis (Izumi et al., 1991). Two days later, the plates were observed with fluorescence microscopy. If the GFP/bsd selection marker was used, green fluorescent plaques were marked with a pen on the bottom of the plates. Alternatively, if the mCherry/bsd selection marker was used, red fluorescent plaques were marked. The plaques were collected by scraping the plaques off from the cell monolayer with pipette tips, followed by aspirating them into the tips. The isolated samples were then transferred into 500 µl D2. The isolated plaques were subjected to three rounds of freeze/thawing, followed by sonication for 20 sec three times. The isolated plaques were used to infect new plates with BSC-1 or BHK-21 cells in 5-fold serial dilutions for one more round of plaque purification in the presence of blasticidin S.

After two rounds of plaque purification with blasticidin S, the plaque purification was performed as above but in a non-selective CMC plaque media without blasticidin S. Here non-fluorescent plaques were marked and isolated. It should be noted that after each round of plaque purification, the isolated plaques were analysed by diagnostic PCRs using primers that amplified the insertion regions. This was to check for the presence of final recombinants and any unwanted wildtype or intermediate recombinant viruses. Plaque purification was repeated until all the isolated plaques contained final recombinants only. The PCR products amplified from the inserted transgenes from the final recombinant viruses were analysed by DNA sequencing to confirm the inserted sequence (Section 2.2.11). The plaques were then used to grow up a recombinant virus stock.

Using this transient dominant selection strategy, multiple VACV recombinants were generated. The more detailed procedures for generating each virus are described below.

2.2.14.1 Isolation of MVA recombinants with transgenes inserted into the VACV TK gene

This method was used to generate MVA recombinants with transgenes inserted into the VACV TK gene region. Transfer plasmids and parental viruses used to generate these recombinant viruses are listed in Table 2-12. All the transfer plasmids used here contain transgenes encoding the minigenes or full-length antigens of interest which were placed under the control of the early/late VACV p7.5 promoter. The transfer plasmids also have a *lacZ* gene encoding β-galactosidase under the control of the late VACV p11 promoter. These DNA sequences are flanked by sequences homologous to the VACV TK gene. There is also a GFP/bsd-encoding sequence in these transfer plasmids, allowing transient dominant selection using blasticidin S to generate final recombinants with transgenes inserted into the VACV TK gene.

Table 2-12| VACV recombinants with transgenes inserted into the VACV TK locus that were generated in this thesis

Recombinant virus generated	Parental virus	Transfer plasmid used
MVA-delTK	MVA	pSC11GB
MVA-TK-SIIN	MVA	pSC11GB-miniOVA
MVA-TK-OVA	MVA	pSC11GB-FullOVA
MVA-Full-PB1F2	MVA	pSC11GB-FullPB1F2
MVA-ESmini-PB1F2	MVA	pSC11GB-ESminiPB1F2
MVA-Full-gB	MVA	pSC11GB-FullgB
MVA-ESmini-gB	MVA	pSC11GB-ESminigB
MVA-delIII-OVA-delTK	MVA-delIII-OVA	pSC11GB
MVA-delIII-SIIN-delTK	MVA-delIII-SIIN	pSC11GB

During the selection process, the first two rounds of plaque purification in the presence of blasticidin S were performed as stated above. However, during the plaque purification process without the selection pressure, agarose plaque media was used instead of CMC plaque media. In brief, confluent BHK-21 cells in 6-well plates were infected with viruses isolated from a previous round of

purification at 37°C with 5% CO_2. After 2 h incubation, the inoculum in each well was replaced with 2 ml agarose plaque media. The plates were incubated for a day at 37°C with 5% CO_2. One day later, 1 ml agarose overlay media with 0.04% X-gal was laid on top of the agarose plaque media. The plates were incubated for one more day. Non-fluorescent plaques that were blue in colour, indicating the existence of β-galactosidase-expressing recombinant viruses, were collected. The insertion sites were verified by diagnostic PCRs during the plaque purification process. The conditions of the PCRs were shown in Table 2-13.

Table 2-13| The conditions of the diagnostic PCRs performed during the isolation of recombinant VACVs with insertions in the TK region

Amplified viral genome region	Primer pair [1]	Size of the PCR product[2]
Wildtype TK region	1. VACV092fwd 2. VACV095rev	1892 bp
GFP/bsd selection marker in the intermediate recombinant VACVs	1. SeqGFPbsd600For 2. revGFPbsdend	568 bp
Left arm of the insertion site in the TK locus in the final recombinant VACVs	1. VACV092fwd 2. laczpSC11rev	1015 bp
Right arm of the insertion site in the TK locus in the final recombinant VACVs	1. SP6 2. TKLrev1 [3]	1019 bp

1 Sequences of the primers are shown in Table 2-4.

2 PCRs were performed with *Taq* DNA polymerase as described in Section 2.2.1. During the extension cycles, the annealing temperature was 55°C and the extension time was 2 min.

3 For MVA-Full-gB, primer FwdHSV-gBend was used instead of the primer SP6 (a 1167 bp product was expected to be produced from MVA-Full-gB).

2.2.14.2 Isolation of MVA recombinants with transgenes inserted into the intergenic region between A11R and A12L genes

This method was used to generate MVA-A11/A12-SIIN and MVA-A11/A12-OVA from wildtype MVA using transfer plasmids P7.5GB-insp7.5miniOVA and P7.5GB-insp7.5OVA respectively. The two transfer plasmids used contain DNA fragments encoding either OVA-257 minigene or full-length OVA under the control of VACV p7.5 promoter, which are flanked by sequences homologous to the VACV A11R and A12L regions. There is a GFP/bsd-encoding sequence in the

transfer plasmids, allowing transient dominant selection of the final recombinants with blasticidin S. The plaque purification process was performed as stated in Section 2.2.14. Diagnostic PCRs were performed to verify the insertion during the plaque purification process (Table 2-14).

Table 2-14| The conditions of the diagnostic PCRs performed during the isolation of recombinant VACVs with insertions in the A11R/A12L intergenic region

Amplified viral genome region	Primer pair	Size of the PCR product [1]
A11R/A12L intergenic region	1. WR130fwd 2. WR131rev	Wildtype virus: 898 bp MVA-A11/A12-SIIN: 1313 bp MVA-A11/A12-OVA: 2561 bp
GFP/bsd selection marker in the intermediate recombinant VACVs	1. SeqGFPbsd600For 2. revGFPbsdend	568 bp
Left arm of the inserts in the A11R/A12L region in the final recombinant VACVs	1. WR130fwd 2. SeqGbF11	718 bp
Right arm of the inserts in the A11R/A12L region in the final recombinant VACVs	1. SP6 2. WR131rev	499

1 PCRs were performed with *Taq* DNA polymerase as described in Section 2.2.1. During the extension cycles, the annealing temperatures was 51°C and the extension time was 1 min.

2.2.14.3 Isolation of WR-eGFP-STS-A3

WR-eGFP-STS-A3 was generated from wildtype WR with the transfer plasmid pSSmCB-WR-EGFP-STS-A3. This plasmid allowed the insertion of DNA sequence encoding the eGFP-STS recombinant antigen directly after the start codon of A3L gene. The selection marker on this plasmid is the mCherry/bsd fusion protein.

The procedures of plaque purification stated in Section 2.2.14 were followed with some modifications. During the first two rounds of plaque purification with blasticidin S selection, plaques that fluoresced both red and green were selected. This ensured that the selected intermediate viruses contained DNA sequences

encoding the eGFP-STS antigen and the mCherry/bsd marker within their viral genomes. During the purification process without blasticidin S, only green fluorescent plaques were selected for further testing and purification. Diagnostic PCRs were performed to verify the insertion and the conditions were illustrated in Table 2-15.

Table 2-15| The conditions of the diagnostic PCRs performed during the isolation of WR-eGFP-STS-A3

Amplified viral genome region	Primer pair	PCR conditions [1]	Size of the PCR product
A3L locus	1. before_A3L_seq 2. rev_A3_454seq	54°C 2 min	Wildtype virus: 902 bp WR-eGFP-STS-A3: 1706 bp
Left arm of the insert from the final recombinant	1. for_eGFP_524seq 2. rev_A3_454seq	57°C 2min	752 bp
Right arm of the insert from the final recombinant	1. before_A3L_seq 2. rev_eGFP_seq	54°C 2 min	502 bp

1 PCRs were performed *Taq* DNA polymerase as described in Section 2.2.1. The annealing temperatures for the primers to the DNA templates and the extension times were shown.

2.2.14.4 Isolation of WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4

Recombinant viruses WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4 were generated from wildtype WR or MVA with transfer plasmids pSSmCB-WR-eGFP-SKS-A4 and pSSmCB-MVA-eGFP-SKS-A4 respectively. These plasmids allowed the insertion of DNA sequence encoding the eGFP-SKS antigen at the 5' end of the A4L gene of WR or MVA. The selection marker encoded by these two plasmids is the mCherry/bsd protein. As such, the procedures of plaque purification stated in Section 2.2.14.3 were followed. Diagnostic PCRs were performed to verify whether the DNA sequence encoding the eGFP-SKS antigen was successfully inserted into the 5' end of A4L gene. The PCR conditions were shown in Table 2-16.

Table 2-16| The conditions of the diagnostic PCRs performed during the isolation of WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4

Amplified viral genome region	Primer pair	PCR conditions[1]	Size of the PCR product[2]
A4L locus	1. revWR123seq 2. fwdWR124seq	54°C 2 min	Wildtype virus: 966 bp WR-eGFP-STS-A4: 1770 bp
Left arm of the insert from the final recombinant	1. for_eGFP_524seq 2. revWR123seq	54°C 2 min	794 bp
Right arm of the insert from the final recombinant	1. SeqRevpEGFPN1 2. fwdWR124seq	57°C 2min	524 bp

1 PCRs were performed as described in Section 2.2.1. The annealing temperatures for the primers to the DNA templates and the extension times were shown.

2 DNA fragments in slightly different sizes were produced during the generation of MVA-eGFP-SKS-A4. The first primer set produced PCR products in size of 939 bp (wildtype MVA) or 1743 bp (MVA-eGFP-SKS-A4). The second and the third primer sets produced DNA fragments in sizes of 767 and 524 bp respectively.

2.2.15 Viral DNA preparation from virus stocks or from isolated plaques

Viral DNA prepared from pure virus stocks was used as the template for PCR for amplifying viral sequences of interest (Section 2.2.1). 10 µl of virus stock was mixed with 10 µg/ml proteinase K (Sigma-Aldrich) diluted in 1× PCR ThermoPol buffer (New England Biolabs) in a final volume of 200 µl. The virus solution was incubated at 56°C for 20 min. Proteinase K was then heat inactivated at 85°C for 10 min. The sample was then used as a DNA template for PCR.

Viral DNA were also prepared from the plaques isolated during the process of recombinant virus generation for diagnostic PCRs. Briefly, 96-well plates of confluent BSC-1 or BHK-21 cells were incubated with 20-40 µl viral solution from individual isolated plaques. 100 µl of fresh D10 was added on top. Plates were incubated at 37°C in 5% CO_2 for two days. Media were then removed and the infected cell monolayers were washed with 150 µl of PBS. 100 µl of 10 µg/ml proteinase K solution in 1× PCR ThermoPol buffer was added to each well. The plates were frozen at -80°C and then thawed at room temperature to lyse the cell membranes to release viruses. The plates were incubated at 56°C for 20 min and then 85°C for 10 min. The supernatants were used as DNA templates for PCR.

2.2.16 Preparation of virus stock

2.2.16.1 Preparation of a virus seed stock

To prepare a virus seed stock, a 25cm^2 flask of confluent BHK-21 cells was inoculated with 5ml of D2 containing 200-300 µl of an isolated plaque, which had been confirmed to contain the final recombinant virus. After three days of incubation at 37°C in 5% CO_2, the infected cells were scraped from the flask with a cell lifter (Corning). Infected cells were collected by centrifugation at 2,000 revolutions per minute (rpm) for 10 min (Beckman Coulter AllegraX-15R). The supernatant was removed and the cell pellet was resuspended in 500 µl of 10 mM Tris (pH 9). The resuspended solution was subjected to three rounds of freeze/thawing, followed by sonication for 20 sec three times. This crude viral seed stock was titrated as in Section 2.2.17.

2.2.16.2 Preparation of a crude virus stock

To prepare a crude virus working stock, one or two 175cm^2 flasks of confluent BHK-21 cells were each inoculated with 30 ml of D2 media containing the master virus stock (Section 2.2.16.3) at an m.o.i. of 0.1 and incubated at 37°C in 5% CO_2 for three days. Infected cells were harvested by scraping the flasks with cell lifters, followed by centrifugation at 2,000 rpm and 4°C for 10 min (AllegraX-15R). Cell pellets were resuspended in 1-2 ml of 10 mM Tris buffer (pH 9). The resuspended infected cells were subjected to three rounds of freeze/thawing, followed by sonication for 20 sec three times. This crude viral stock was titrated as in Section 2.2.17.

2.2.16.3 Preparation of a semi-purified virus working stock via sucrose cushion centrifugation

To prepare a semi-purified working virus stock from a newly prepared seed stock, 5-10 175cm^2 flasks of confluent BHK-21 cells were each inoculated with 30 ml of D2 media containing the crude virus stock at an m.o.i. of 0.1 at 37°C in 5% CO_2 for three days. The infected cells were then harvested by scraping the flasks with cell lifters, followed by centrifugation at 2,000 rpm and 4°C for 10 min (AllegraX-15R). Cell pellets were resuspended in 5-10 ml of 10 mM Tris buffer (pH 9.0). The cells were homogenised in a Dounce homogeniser (Wheaton) with

60 strokes to lyse the cell membrane to release viruses. The homogenates were centrifuged at 800 rpm at 4°C for 5 min (AllegraX-15R) to remove nuclei. Supernatants were collected. The nuclei pellets were resuspended in 5-10 ml of 10 mM Tris (pH 9) and were re-homogenised and centrifuged again to release more viruses. The supernatants were pooled. To prepare the sucrose cushion, 17 ml of 36% sucrose in 10 mM Tris (pH 9) was first added to ultracentrifuge tubes (Beckman Coulter). 5 ml of the virus supernatant was layered evenly on top of each sucrose cushion solution, and then topped up with 10 mM Tris (pH 9). In general, two to four sucrose cushions were prepared for each virus stock. The samples were then centrifuged at 13,500 rpm for 80 min at 4°C in a Beckman Coulter Optima LE-80K Ultracentrifuge with a SW-32Ti rotor. Supernatants were aspirated and the pellets, which contained mostly purified viruses, were resuspended in 1-2 ml of 10 mM Tris (pH 9). The resuspended solution was used as a working virus stock and was titrated before use (Section 2.2.17).

2.2.16.4 Preparation of a purified virus working stock via sucrose gradient centrifugation

To prepare a purified working virus stock, sucrose gradient centrifugation was used. In brief, two sets of 24% to 40% continuous sucrose gradient were prepared in sterile ultracentrifuge tubes by layering 6 ml each of 40%, 36%, 32%, 28% and 24% sucrose in 10 mM Tris (pH 9). The tubes were stored at 4°C overnight. One day later, a semi-purified virus stock was prepared by sucrose cushion purification (Section 2.2.16.3) and was resuspended in 10 mM Tris (pH 9). The virus solution was sonicated twice for 30 sec and was layered on top of a sucrose gradient. The sample was then centrifuged at 12,000 rpm for 50 min at 4°C in the Optima LE-80K Ultracentrifuge. After centrifugation, the milky layer, which contained purified VACV, near the middle of the tubes was collected with careful pipetting and was transferred to a sterile 50 ml tube (BD Biosciences). The remaining supernatant was discarded. The pellet at the bottom of the sucrose gradient contained aggregated viruses. It was resuspended in 10 mM Tris (pH 9), sonicated twice for 30 sec, and layered on top of the remaining sucrose gradient. The samples were centrifuged at 12,000 rpm for 50 min at 4°C again in the Optima LE-80K Ultracentrifuge. After the centrifugation, the milky VACV layer was

collected and pooled with the purified virus collected before. The virus solution was transferred into two sterile ultracentrifuge tubes in equal volume. The tubes were topped up with 10 mM Tris (pH 9) and were then centrifuged at 13,500 rpm for 60 min at 4°C (Optima LE-80K Ultracentrifuge). The purified virus pellets were resuspended in 1-2 ml of 10 mM Tris (pH 9). The sample was titrated before use (Section 2.2.17).

2.2.17 Virus titration

Titration of VACV was performed by plaque assay to ensure that a known amount of virus was used in each experiment. For viruses on the VACV WR background, their titers were determined with confluent BSC-1 cell monolayers in 6-well plates. For viruses on the strain MVA background, their titers were determined with confluent BHK-21 cell monolayers grown in 6-well plates.

2.2.17.1 Titration of VACV strain WR

Duplicate 10-fold serial dilutions of virus stock were prepared in D0 media. Media was removed from two 6-well plates with confluent BSC-1 cell monolayers and 0.5 ml of the virus dilutions were added to wells and incubated for 90 min at 37°C with 5% CO_2. During the incubation, plates were rocked every 10-15 min. After 90 min of incubation, the virus inocula were aspirated, and the cells were overlaid with warm CMC plaque media. The cells were incubated at 37°C and 5% CO_2 for three days. Three days later, the media were aspirated and the plaques were visualized by staining with crystal violet staining solution. The number of plaques was counted and the titer in plaque-forming unit (PFU)/ml was calculated according to the virus dilution used and was averaged from the duplicate.

2.2.17.2 Titration of VACV strain MVA

Duplicate 10-fold serial dilutions of a MVA stock were prepared and used to inoculate confluent BHK-21 cell monolayers in 6-well plates, as in the titration of WR described in Section 2.2.17.1. After 90 min of inoculation with virus dilutions, inocula were removed and replaced with warm D2 media. The plates were incubated at 37°C with 5% CO_2 for one day. Cell monolayers were then

fixed with a 1:1 solution of methanol:acetone for 5 min. The fixing solution was removed and the monolayers were washed with 2 ml PBS-2%FBS twice, and then incubated for 1 h at room temperature with 1 ml rabbit polyclonal anti-MVA antiserum (diluted in 1 in 1,000 in PBS-2%FBS). The cell monolayers were then washed with 2 ml PBS-2%FBS twice and were incubated with 1 ml anti-rabbit-IgG-peroxidase (diluted in 1 in 500 in PBS-2%FBS). Cell monolayers were washed twice with PBS-2%FBS and incubated with 0.8 ml DAB substrate solution. Areas of blue-black colour were formed on cell monolayers, indicating foci of cells infected with MVA. The number of stained foci was counted. The titer in PFU/ml was calculated according to the virus dilution used and was averaged from the duplicate.

2.2.17.3 VACV titration from isolated organs

Organs were taken from mice immunised with WR or WR recombinants (Section 2.2.18) at times indicated. The organs were stored at -80°C before use. Samples were thawed, and homogenised with 1ml tissue grinders (Wheaton). The homogenates were resuspended in a final volume of 1 ml in D0 media. The samples were frozen and thawed three times, followed by sonication for 20 sec three times. VACV titers were determined by plaque assays on BSC-1 cells as described in Section 2.2.17.1.

2.2.18 Immunisation of mice

2.2.18.1 i.d. immunisation

Mice were anesthetised by isoflurane inhalation (4%, in oxygen with a flow rate of 800 ml/min) using an anaesthetic machine (Advanced Anaesthesia Specialists) (Lin et al., 2012). The anesthetised mice were injected with 10 µl virus inocula diluted in PBS into ear pinnae using 100 µl Gastight glass syringes (Hamiton) and 27-gauge needles (BD Biosciences). For in vivo cross presentation experiments, 2×10^6 cells resuspended in 20 µl PBS were injected into ear pinnae of the anesthetised mice. In some experiments, lesions formed on the infected ear pinnae were measured with a vernier calliper (Trojan).

2.2.18.2 i.p. immunisation

Mice were injected with 200 µl of virus or cell inocula resuspended in PBS using 1 ml tuberculin syringes (BD Biosciences) and 27-gauge needles into the peritoneal cavity of the mice.

2.2.19 Preparation of splenocytes from mice

Naive or immunised mice were culled and spleens were collected. The spleens were homogenised using plungers from 1 ml tuberculin syringes and the splenocytes were filtered through 70 µm cell strainers (BD Biosciences) by washing with PBS. Single cell suspensions of splenocytes from each mouse were centrifuged at 1,500 rpm at 4°C for 5 min (AllegraX-15R) and were resuspended in 5 ml red cell lysis buffer for 3 min. 30 ml PBS was added to each sample, mixed well, and centrifuged at 1,500 rpm at 4°C for 5 min (AllegraX-15R). The splenocytes were resuspended in 5 ml D10. Viable splenocytes were counted after staining with trypan blue solution using a haemocytometer or a Countess automated cell counter (Life Technologies).

2.2.20 Isolation of purified F5 TCR transgenic $CD8^+$ T cells

The $CD8\alpha^+$ T cell isolation kit II (Miltenyi Biotec) was used to isolate $CD8^+$ T cells from F5 TCR transgenic mice by selective depletion of other cell types. The manufacturer's instructions were followed. Briefly, splenocytes from naive F5 mice were resuspended in MACS buffer. The cells were centrifuged at 1,500 rpm at 4°C for 10 min (AllegraX-15R). The supernatant was aspirated and the cells were labelled with the biotin-antibody cocktail provided in a ratio of 10 µl/10^7 cells. The solution was mixed and incubated at 4°C for 10 min. The anti-biotin microbead solution provided was then added to the sample in a ratio of 20 µl/10^7 cells. The solution was incubated at 4°C for 15 min. The labelled cells were then washed with MACS buffer and were resuspended in 1 ml fresh MACS buffer. The labelled cells were passed over a magnetised LS column (Miltenyi Biotec). The column was washed three times with MACS buffer. All the flow-through solutions containing untouched $CD8^+$ T cells were collected and pooled together. The pooled solution was then centrifuged at 1,500 rpm at 4°C for 5 min

(AllegraX-15R) and the cell pellet was resuspended in PBS. The cells collected were untouched purified $CD8^+$ T cells (at around 95% purity).

2.2.21 Generation of BMDCs

BMDCs from C57Bl/6 mice were prepared as described by Lutz et al. (1999) with some modifications. In brief, mice were culled and femurs and tibiae were collected. Both ends of the bones were cut and the bone marrow was flushed out with D0 media using 23 gauge needles (BD Biosciences) attached to 10 ml syringes (BD Biosciences). The bone marrow was centrifuged at 1,500 rpm at 4°C for 5 min (AllegraX-15R) and resuspended in D10 media. Cell aggregates were broken up by pipetting. Cells were counted and resuspended at 1×10^6 cells/ml in the DC media. 5×10^6 cells were placed into individual wells of 6-well plates. The cells were incubated at 37°C with 5% CO_2 for 9 days. On days 3, 6, and 8, 2.5 ml of the media from each well were collected and centrifuged at 1,500 rpm at 4°C for 5 min (AllegraX-15R). Cell pellets were resuspended in fresh DC media (2.5 ml for each well), and added back to the 6-well plates. At day 9, non-adherent and easily-dislodged cells were resuspended in the 6 well-plates by gentle pipetting. The cells were collected and resuspended in D10 media. The cells were counted and stored on ice before use.

2.2.22 Adoptive transfer of immune cells into mice

Cells for adoptive transfer were resuspended in 200 µl PBS at concentrations as stated below. Mice were placed under an infrared lamp for 2 min and were then put inside in a restraining device with tails out. 200 µl of cells were injected into lateral tail veins with 0.5 ml insulin syringes (with 27-gauge needles, Terumo). For $CD8^+$ T cell transfer, 5×10^3 or 1×10^6 cells were injected into each mouse. For immunisation with peptide-pulsed BMDCs, mice were injected with 2×10^6 BMDCs. For in vivo cytotoxicity assays, 1×10^6 peptide-pulsed splenocytes were adoptively transferred into mice.

2.2.23 CpG and cytc treatments

Mice were placed under an infrared lamp for 2 min and were then put inside in a restraining device with tails out. Mice were then injected i.v. via tail veins with 20

nmol synthetic phosphorothioated CpG1668 oligonucleotide diluted in 200 µl PBS one day before immunisation with 0.5 ml insulin syringes (27-gauge needles). For cytc treatment, mice were injected i.v. with 5 mg horse cytc in 200 µl PBS for five consecutive days starting one day before immunization. Control mice were injected with 200 µl PBS.

2.2.24 VACV infection of cells in vitro

2.2.24.1 Generation of antigen donor cells for in vivo cross priming assays

293A cells in single cell suspension were incubated with viruses indicated at an m.o.i. of 5 for 1 h at 37°C in a shaking incubator (shaking at 200 rpm) in a volume of 0.5-2 ml of D0, depending on the number of cells used. Infected cells were then transferred to warm 40 ml D2 media in 50 ml tubes and the infection were continued for another 5 h at 37°C with gentle rotation (4 rpm, MACSmix tube rotator (Miltenyi Biotec) in a 37°C incubator).

2.2.24.2 Infection of cells for in vitro antigen presentation

293-D^b, 293-K^b, DC2.4 or BMDCs (generated as described in Section 2.2.21) in single cell suspension were mixed with viruses indicated at an m.o.i. of 5 in a volume of 0.5 ml in D0 media. The cells were then incubated at 37°C with shaking at 200 rpm in a shaking incubator for 1 h. Infected cells were then transferred to 10 ml warm D2 media in 15 ml tubes and the incubation was continued for another 5 h at 37°C. During the infection period, the cells were either rotated at 4 rpm using the MACSmix tube rotator or were gently mixed every 20 min. Aliquots of cells were taken at times indicated and were stored on ice before further use.

2.2.24.3 In vitro growth analysis of VACV recombinants

Confluent BSC-1 cell monolayers in 6-well plates were infected with viruses indicated at an m.o.i. of 0.01 in 1ml D2 media. Plates were incubated for 1 h at 37°C with 5% CO_2. Infectious inocula were aspirated, cells were washed with 1 ml D2, and 2 ml D2 were added. The plates were incubated at 37°C with 5% CO_2 for the time indicated. To harvest infectious viruses from the infected cells, cell monolayers were scraped, followed by centrifugation at 2,000 rpm for 10 min.

Supernatant was removed and cells were resuspended in 1 ml D2 media. Cells were then subjected to three rounds of freeze/thawing cycle to release viruses. The amount of infectious viruses was determined by plaque assay (Section 2.2.17).

2.2.25 TMP/UV and AMT/UV treatment of VACV or VACV-infected cells

Two psoralen derivatives, namely TMP and AMT, were tested for their ability to inhibit VACV replication and viral gene expression. Solutions of VACV or VACV-infected cells containing 10 μg/ml TMP or 100 μg/ml AMT in PBS were prepared. The samples were transferred to small plastic weighing trays that had been wrapped with parafilm (Pechiney plastic packaging). The whole setup was put on top of ice. The samples were irradiated 10 mm below a 365 nm UV-A lamp (Vilber Lourmat) for up to 60 min. The samples were mixed by pipetting every 8-10 min. Aliquots of samples were taken at times indicated.

To block VACV replication but still allow early virus gene expression, VACV inocula containing 1 μg/ml TMP in PBS were prepared. The samples were irradiated 10 mm below a 365 nm UV-A lamp for 3 min. The samples were mixed every 30 sec during the irradiation.

2.2.26 Heat inactivation of VACV or VACV-infected cells

Heat inactivation was used to inhibit virus replication and viral gene expression of VACV inocula or of residual viruses on VACV-infected cells. In brief, VACV or VACV-infected cells were resuspended in PBS (maximum volume: 1 ml). The solutions were then heat-treated in a 60°C heat block for 60 min.

2.2.27 In vitro activation of F5 TCR transgenic $CD8^+$ T cells

Half of the splenocytes prepared from naive F5 TCR transgenic mice were taken, centrifuged at 1,500 rpm at 4°C for 5 min (AllegraX-15R), and resuspended in 2.5 ml of 0.1 μM synthetic NT60NP-366 peptide diluted in D0 media. The cells were incubated at 37°C for 1 h in a shaking incubator (shaking at 200 rpm) for peptide-pulsing. The peptide-pulsed cells were washed twice with D10 media and mixed with the unpulsed cells. The mixed cells were counted, centrifuged at 1,500 rpm at 4°C for 5 min (AllegraX-15R), and resuspended in 2.5×10^6 cells/ml in T cell

media supplemented with 1.25 ng/ml recombinant mouse IL-2 (R&D System). Cells were incubated in 75 cm^2 culture flasks at 37°C with 5% CO_2. One day later, cells were pelleted by centrifugation at 1,500 rpm for 5 min at 4°C (AllegraX-15R). The cells were resuspended at 2.5×10^6 cells/ml in T cell media supplemented with 2.5 ng/ml recombinant mouse IL-2. The cells were incubated at 37°C with 5% CO_2 overnight, and an equal volume of fresh T cell media with 2.5 ng/ml recombinant mouse IL-2 was added to the cells. The cells were further incubated at 37°C with 5% CO_2 for another day before use.

2.2.28 Detection of OVA-257/H-2K^b presentation on APCs

$0.2\text{-}1\times10^6$ cells were resuspended in 20 µl of the culture supernatant of hybridoma 2.4G2 (to block Fc receptors) for 20 min at 4°C. Cells were washed with PBS-2% FBS, and then resuspended in 50 µl 25D1.16-APC antibody (1 in 150 dilution) in PBS-2%FBS. The cells were incubated at 4°C for 30 min. The antibody-labelled cells were washed once with PBS-2%FBS, followed by a wash with PBS. Cells were then fixed with 50 µl 1% paraformaldehyde in PBS at room temperature for 20 min. Cells were washed twice with PBS-2%FBS. The cells were resuspended in 50-100 µl PBS-2%FBS and were ready for flow cytometric analysis.

2.2.29 Re-stimulation of activated CD8$^+$ T cells

2.2.29.1 Re-stimulation with synthetic peptides

Splenocytes from immunised mice (Sections 2.2.18 and 2.2.19) or in vitro-activated CD8$^+$ T cells (Section 2.2.20) were isolated and were adjusted to 1×10^7 cell/ml in D10 media. 100 µl of the cells were transferred to wells of 96-well plates (U-bottom, BD Biosciences). Unless otherwise stated, 100 µl of 0.2 µM synthetic peptides in D0 media were added to individual wells. Therefore, cells were generally co-cultured with 0.1 µM synthetic peptides. After 1 h of incubation at 37°C with 5% CO_2, 20 µl of 50 µg/ml brefeldin A diluted in D10 media was added to each well. The cells were further incubated for another 3 h.

In some cases, synthetic peptides were titrated against splenocytes isolated from immunised mice. Here, 10-fold serial dilutions of peptides were prepared with D0 media. The peptide dilutions were then added to the splenocytes as stated above.

2.2.29.2 Re-stimulation with infected APCs

Instead of synthetic peptides used in Section 2.2.29.1, infected cells (as prepared in Section 2.2.24.2) were used as stimulators here. In brief, 1×10^6 splenocytes or activated $CD8^+$ T cells in 100 µl D10 media were co-cultured with infected cells resuspended in 100 µl D2 media in wells of 96-well plates (U-bottom). A stimulator: effector ratio of 1:5 was normally used, unless otherwise stated. The cells were incubated at 37°C with 5% CO_2 for 1 h. 20 µl of 50 µg/ml brefeldin A in D10 was then added to the cells. The cells were incubated for another 3 h.

2.2.30 Intracellular cytokine staining (ICS)

In most experiments, only intracellular IFN-γ staining was performed. Briefly, after re-stimulation of splenocytes or activated $CD8^+$ T cells as stated in Section 2.2.29, the cells were centrifuged at 2,100 rpm at 4°C for 3 min (AllegraX-15R). Media were removed and cells were resuspended in 40 µl anti-CD8α-PE (1 in 150 dilution in PBS-2%FBS). Cells were incubated at 4°C in the dark for 30 min. Cells were washed with PBS-2%FBS, followed by an additional wash with PBS. The cells were fixed in 40 µl 1% paraformaldehyde in PBS at room temperature for 20 min. Cells were washed with PBS-2%FBS three times and then stained with 40 µl anti-IFN-γ-APC antibody (1 in 200 dilution) in 0.25% saponin diluted in PBS-2%FBS at 4°C overnight in the dark. The next day, cells were washed three times with PBS-2%FBS and were resuspended in 50 µl of PBS-2%FBS. The cells were ready for analysis by flow cytometry.

There were two variations to the standard ICS described above. The first variation involved co-labelling of IL-2, TNF-α and IFN-γ simultaneously. Here, anti-IL-2-PE-Cy5 and anti-TNF-α-PE-Cy7 antibodies were used in addition to anti-IFN-γ-APC during the intracellular cytokine staining. The second variation involved tracking responses induced by the transferred $CD45.2^+$ $CD8^+$ F5 cells. Here, anti-CD45.2-FITC antibody was added in addition to anti-CD8-PE during the cell surface antibody staining step.

2.2.31 DimerX labelling

DimerX staining was performed according to the manufacturer's instructions. In brief, 2 μg of H-2Kb:Ig fusion protein was incubated overnight at 37°C with a 160 molar excess of a synthetic peptide in PBS. The peptide-loaded dimers were then incubated with 0.8 μg of the secondary anti-mouse-IgG1-PE antibody at room temperature for 1 h in the dark. This allowed the binding of two peptide-loaded DimerX:Ig fusion proteins with one bivalent secondary antibody. 1×10^6 splenocytes were first incubated with 20 μl of the culture supernatant of hybridoma 2.4G2 for 15-20 min at 4°C to block Fc receptors on the cells. Cells were then washed with PBS-2% FBS and were labelled with the peptide-loaded dimers and anti-CD8α-APC (1 in 150 dilution) in 50 μl PBS-2%FBS for 1 h on ice. The labelled cells were washed twice with PBS-2%FBS. Cells were reuspended in 50-100 μl PBS-2%FBS and were ready for flow cytometric analysis.

2.2.32 CFSE labelling

CFSE labelling of cells was required for in vivo $CD8^+$ T cell proliferation (Section 2.2.33.2) and in vivo cytotoxicity assays (Section 2.2.35). Cells were resuspended in 2 ml D0 media containing CFSE at the concentrations stated below. The cells were incubated in a 37°C water bath for 10 min with gentle shaking every 2-3 min. After the incubation, cold D10 was added to stop the labelling reaction. Cells were then washed with D10 media three times. After the last wash, cells were resuspended in PBS at the appropriate concentration.

For an in vivo proliferation assay (Section 2.2.33.2), purified TCR transgenic $CD8^+$ T cells were labelled with 5 μM CFSE. For an in vivo cytotoxicity assay (Section 2.2.35), peptide-pulsed splenocytes were labelled with 0.25 μM CFSE while unpulsed splenocytes were labelled with 2 μM CFSE.

2.2.33 In vivo early activation and proliferation assay

2.2.33.1 Early activation assay based on CD69 expression

B6.SJL mice were adoptively transferred with 1×10^6 F5 $CD8^+$ T cells via i.v. injection. One day later, mice were immunised with recombinant viruses as

indicated. One day post immunisation, splenocytes were isolated. 1-2×10^6 splenocytes were stained with 50 µl PBS-2%FBS containing anti-CD8α-PE, anti-CD45.2-APC and anti-CD69-PE-Cy7 antibodies (each in 1: 150 dilution) for 30 min at 4°C. The cells were then washed once with PBS-2%FBS, followed by a wash with PBS. Cells were fixed with 50 µl 1% paraformaldehyde in PBS at room temperature for 20 min. Cells were washed twice with 150 µl PBS-2%FBS and resuspended in 80-100 µl PBS-2%FBS. The cells were ready for flow cytometric analysis.

2.2.33.2 In vivo CFSE-based proliferation assay with CD25 straining

B6.SJL mice were adoptively transferred with 1×10^6 F5 $CD8^+$ T cells labelled with 5 µM CFSE. One day later, mice were immunised with recombinant viruses as indicated. Two days post immunisation, splenocytes were isolated. 1-2×10^6 splenocytes were stained with 50 µl PBS-2%FBS containing anti-CD8α-APC-Cy7, anti-CD45.2-APC and anti-CD25-PE antibodies (each in 1: 150 dilution) for 30 min at 4°C. The cells were then washed once with 150 µl PBS-2%FBS and were resuspended in 50 µl of PBS+2%FBS containing 0.04 µg/µl 7-amino actinomycin D (7AAD; Life Technologies). 7AAD binds to the DNA of dead cells and fluoresces, allowing discrimination of live and dead cells during flow cytometric analysis. Cells were incubated at 4°C for 30 min. Cells were then washed once with PBS+2%FBS, followed by a wash with PBS. Cells were fixed with 50 µl 1% paraformaldehyde in PBS containing 5 µg/ml actinomycin D (Sigma-Aldrich) at room temperature for 20 min. Cells were washed twice with PBS-2%FBS and resuspended in 80-100 µl PBS-2%FBS. The cells were ready for flow cytometric analysis.

2.2.34 Peptide-pulsing of BMDCs

5×10^6 BMDCs (Section 2.2.21) were collected and centrifuged at 1,500 rpm at 4°C for 5 min (AllegraX-15R). Cells were resuspended in 2.5 ml D0 with 1 µM synthetic HBcAg-128 peptide, together with either 1 µM PR8NP-366 or A47-171 peptides. Cells were incubated at 37°C for 60 min in a shaking incubator (shaking at 200 rpm). After the incubation, cells were centrifuged at 1,500 rpm at 4°C for 5

min (AllegraX-15R), and resuspended in 500 µl PBS. The peptide-pulsed cells were used for i.v. injection into mice (Section 2.2.22).

2.2.35 CFSE-based in vivo cytotoxicity

A single cell suspension of splenocytes was divided into two populations. One population was pulsed with 0.1 µM synthetic gB-498 peptide in the presence of Vybrant DiD cell-labelling dye (in 1 in 200 dilution; Life Technologies) in D0 media at 37°C for 1 h in a shaking incubator (200 rpm shaking). The second population was left unpulsed but was also stained with 1 in 200 diluted Vybrant DiD dye in D0 media. The two populations were then washed twice with D10, followed by a wash with D0. The peptide-pulsed and unpulsed populations were labelled with 0.25 µM and 2 µM CFSE in D0 as stated in Section 2.2.24. The cell numbers were counted and the two populations were mixed together in a 1:1 ratio. Cells were resuspended in 5×10^7 cells/ml in PBS. 1×10^7 mixed cells were i.v. injected into naive or immunised mice via the tail vein (Section 2.2.19). Four hours after cell transfer, the spleens from the mice were collected and single splenocyte suspensions were prepared. The samples were subjected to flow cytometric analysis. The transferred cell population, which is DiD^+, was identified from the collected events during flow cytometric analysis. Within this population, the percentages of $CFSE^{hi}$ (unpulsed) and $CFSE^{lo}$ (gB-498-pulsed) populations were measured. The equation below was used to calculate the specific lysis of the gB-498-pulsed cells:

$$\%\ \text{specific lysis} = \left\{ 1 - \left[\frac{\left(\frac{\text{percentage of } CFSE^{hi}}{\text{percentage of } CFSE^{lo}}\right)_{\text{naive mouse}}}{\left(\frac{\text{percentage of } CFSE^{hi}}{\text{percentage of } CFSE^{lo}}\right)_{\text{immunised mouse}}} \right] \right\} \times 100$$

2.2.36 Data acquisition with flow cytometry

Flow cytometry discriminates individual cells into separated populations based on fluorescence intensities. Here, cells were firstly labelled with fluorescent dyes or with fluorescent-labelled antibodies that bind to proteins of interest as described above. Flow cytometry was then used to determine the percentages of cells expressing proteins of interest and the level of protein expression in individual

cells. Cells labelled with fluorescent dyes were detected similarly. In this study, a LSR-II Flow Cytometer (BD Biosciences) was used for data acquisition. Adequate numbers of events were collected. For instance, 100,000 $CD8^+$ events were collected from each ICS samples in general.

2.2.37 Data analysis

2.2.37.1 Measurement of plaque sizes

Images of plaques from cell monolayers infected with VACV were acquired by the Olympus DP20 digital microscope camera mounted on the Olympus microscope CKX41. The diameters of the plaques were measured from the images using ImageJ 1.47 software (Schneider et al., 2012). The images were then prepared using Photoshop CS2 or CS4 softwares (Adobe).

2.2.37.2 Flow cytometric analysis

Flow cytometry data (Section 2.2.36) were analysed with FlowJo 8.8.4 software (Tree Star). To analyse the data, appropriate gates were applied to the samples as stated below.

(1) ICS (Section 2.2.30)

Events were first gated on a side scatter and forward scatter (SSC×FSC) plot. In this population, the $CD8^+$ population was gated on a SSC × CD8 plot, then the percentage of $CD8^+$ events that were IFN-γ^+ was determined on an IFN-γ × CD8 plot. Backgrounds were determined using controls that were cultured with media only, without any synthetic peptides. The gating scheme is illustrated on Figure 2-1. The final data for each sample was calculated by subtracting the background. The total number of $CD8^+$, IFN-γ^+ T cells was calculated as below:

Total number of $CD8^+$, IFN-γ^+ T cells
= % IFN-γ^+ of $CD8^+$ cells × % $CD8^+$ of all lymphocytes × total splenocyte count

There are two variations on how the events were gated. Firstly, to identify the $CD45.2^+$ adoptively transferred cells that produced IFN-γ after ex vivo peptide stimulation from splenocytes of the B6.SJL recipients, $CD8^+$ events that were

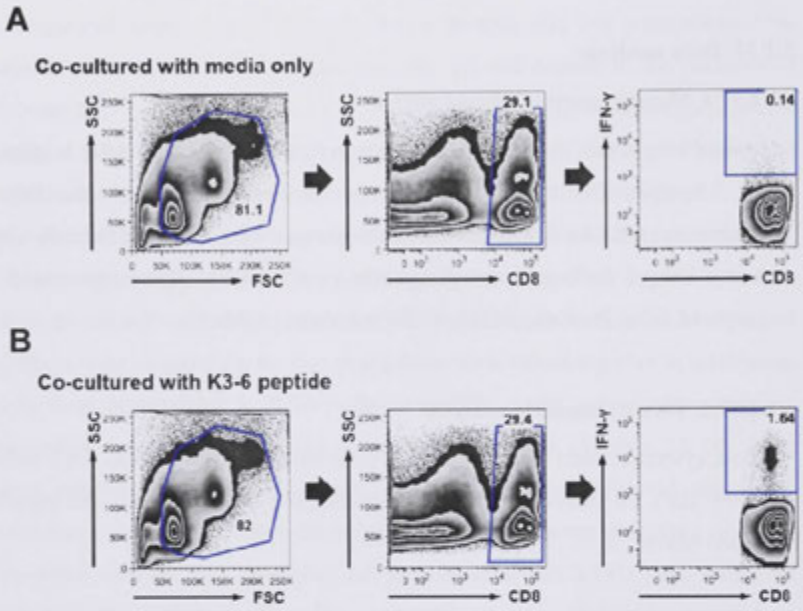


Figure 2-1| The gating strategy for identifying $CD8^+$ IFN-γ^+ cells from splenocytes of immunised mice after ex vivo stimulation with peptides, followed by ICS. Representative flow cytometric plots of splenocytes, from a mouse immunised with VACV WR for seven days, after mock stimulation with media only (without any peptide; A) or ex vivo stimulation with K3-6 peptide (B), followed by ICS. Cells were first gated on SSC × FSC plots. The $CD8^+$ events were then gated from the lymphocyte populations on SSC × CD8 plots. The IFN-γ^+ cells were identified form the $CD8^+$ populations on IFN-γ × CD8 plots. The number in each plot represents the percentage of events of the gated population relative to the population shown in the plot.

$CD45.2^+$ and IFN-γ^+ were determined. This allowed the detection of the frequency of $CD45.2^+$ IFN-γ^+ cells relative to the overall $CD8^+$ population. The percentage of IFN-γ^+ cells within the $CD8^+$ $CD45.2^+$ events was also determined. Secondly, when ICS with IL-2, TNF-α and IFN-γ was performed, the $CD8^+$ events that were positive for individual cytokines were first gated separately. Boolean gating analysis was then conducted to determine the frequency of events that were positive for each of the possible cytokine combination. It should be noted that events that were negative for all cytokines were not included during analysis. Here, SPICE 5.22 software (Roederer et al., 2011) was also used in conjunction with FlowJo for data analysis.

(2) DimerX labelling (Section 2.2.31)

A similar gating method as the one used for ICS (refer to Figure 2-1) was used to identify the $CD8^+$ populations from the samples. In brief, cells were first gated on a SSC × FSC plot, followed by gating on a SSC × CD8 plot to identify the $CD8^+$ population. The percentage of $CD8^+$ events that were $DimerX^+$ was determined on a DimerX × CD8 plot (refer to Figure 5-2C). Backgrounds were determined using DimerX pulsed with an irrelevant peptide. The final data for each sample was calculated by subtracting the background.

(3) 25D1.16 antibody labelling (Section 2.2.28) and GFP expression

Events were first gated on a SSC × FSC plot. From this population, the mean fluorescence intensity (MFI) of the fluorophore-conjugated 25D1.16 antibody on the gated events was determined. A similar gating scheme used for 25D1.16 antibody labelling analysis was used here, except the MFI of GFP in cells was determined.

(5) CD69 expression (Section 2.2.33.1)

Figure 2-2 illustrates the gating scheme for identifying the transferred $CD45.2^+$ $CD8^+$ F5 T cells from the B6.SJL recipients. Briefly, cells were first gated on a SSC × FSC plot. From this population, the $CD8^+$ population was gated on a SSC × CD8 plot. The transferred F5 $CD8^+$ T cell population was identified as $CD45.2^+$ from the $CD8^+$ population on a CD45.2 × CD8 plot. From the $CD45.2^+$ population, a histogram was used to illustrate the level of anti-CD69 labelling on

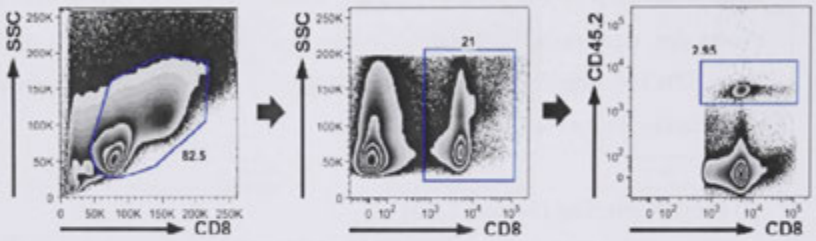


Figure 2-2| The gating scheme for identifying the CD45.2$^+$ CD8$^+$ F5 cells that were adoptively transferred into B6.SJL recipients for the early activation assay based on CD69 expression. A B6.SJL (CD45.1$^+$) mouse was adoptively transferred with purified 1×10^6 CD45.2$^+$ CD8$^+$ F5 cells a day before immunised with a WR recombinant, WR-delTK. One day post immunisation, splenocytes from the mouse were prepared and labelled with anti-CD8α-PE, anti-CD45.2-APC and anti-CD69-PE-Cy7 antibodies by following the procedures shown in Section 2.2.33.1. To identify the transferred CD8$^+$ F5 cells, cells were first gated on a SSC × FSC plot. The CD8$^+$ events were then gated from the on a SSC × CD8 plot. The CD45.2$^+$ cells were identified form the CD8$^+$ population on a CD45.2 × CD8 plot. This CD45.2$^+$ CD8$^+$ population represented the transferred F5 CD8$^+$ T cells. A histogram was then used to illustrate the CD69 expression on the transferred cell population (refer to Figure 5-10). The number in each plot represents the percentage of events of the gated population relative to the population shown in the plot.

the gated events (refer to Figure 5-10). The MFI of CD69 labelling and the percentage of $CD69^+$ events relative to the $CD8^+$ $CD45.2^+$ events was determined.

(6) CFSE-based in vivo proliferation and CD25 expression (Section 2.2.33.2)

A slightly different strategy was used to identify the transferred $CD45.2^+$ $CD8^+$ F5 T cells from the B6.SJL recipients here. First, cells were gated on a SSC × FSC plot. The singlet population was then gated on a plot of width signal of FSC × area signal of FSC. From this singlet population, live events ($7AAD^-$) were identified on a SSC × 7AAD plot. The $CD8^+$ population was then gated from the live events on a SSC × CD8 plot. The transferred F5 $CD8^+$ T cell population ($CD45.2^+$) was identified from the $CD8^+$ population on a CD45.2 × CD8 plot (Figure 2-3). A CD25 × CFSE plot showing the level of CFSE and anti-CD25 labelling on the events of the $CD8^+$ $CD45.2^+$ population were used to determine the percentages of $CD25^+$ events and $CFSE^{lo}$ events relative to the $CD8^+CD45.2^+$ events (refer to Figure 5-11A). The division index, representing the number of cell division for a transferred cell, was also determined from the transferred $CD45.2^+$ population.

(7) In vivo cytotoxicity assay (Section 2.2.35)

Events were gated on a SSC × FSC plot. From this population, the DiD^+ population was identified and gated on a SSC × DiD plot (Figure 2-4). From the DiD^+ population, a histogram was used to illustrate the level of CFSE labelling on the gated events. From this histogram, percentages of $CFSE^{lo}$ and $CFSE^{hi}$ cells were determined and the information was used to calculate the percentage of specific lysis (refer to Section 2.2.35, Figure 6-10A).

2.2.37.3 Statistical analysis

Statistical analysis was performed in Microsoft Excel 2007 software and GraphPad Prism 5 software. In general, when two groups of samples were compared, the two-tailed, unpaired student's T test was performed. One-way analysis of variance with the Tukey post test was performed if more sample groups were compared. The difference between the two tested samples is considered to be statistical significant when $p < 0.05$.

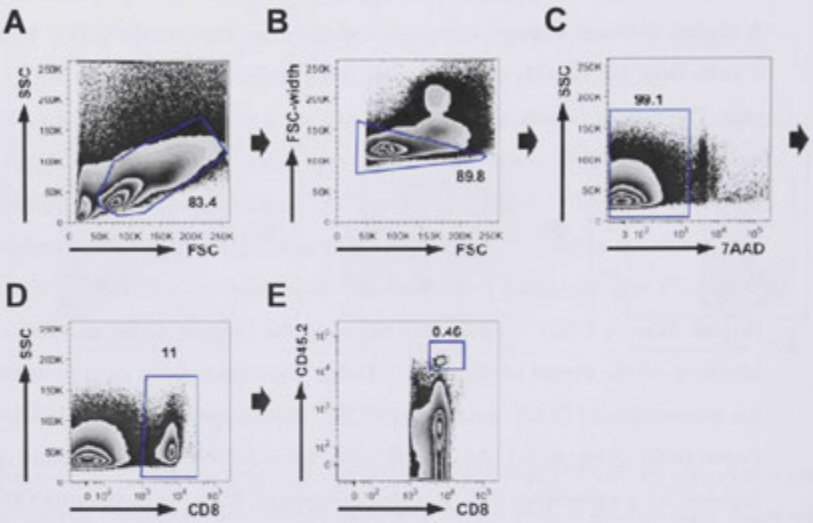


Figure 2-3| Gating strategy for identifying CD45.2$^+$ CD8$^+$ F5 cells that were adoptively transferred into B6.SJL recipients for the in vivo CFSE-based proliferation assay with CD25 straining. A B6.SJL (CD45.1$^+$) mouse was adoptively transferred with purified 1×10^6 CD45.2$^+$ CD8$^+$ F5 cells a day before immunised with a WR recombinant, WR-delTK. Two day after immunisation, splenocytes from the mouse were prepared and were subjected to the procedures described in Section 2.2.33.2. To identify the transferred CD8$^+$ F5 cell population, cells were first gated on a SSC × FSC plot (A). Singlets were gated on a plot of FSC (width signal)× FSC (area signal) (B). Live CD8$^+$ cells were identified on a SSC × 7AAD plot (C), followed by gating on a SSC × CD8 plot (D). The transferred CD45.2$^+$ CD8$^+$ F5 cells were identified from the CD8$^+$ population on a CD45.2 × CD8 plot (E). A CD25 × CFSE plot was then used to illustrate the CD25 expression level and CFSE level in the transferred cell population (refer to Figure 5-11). The number in each plot represents the percentage of events of the gated population relative to the population shown in the plot.

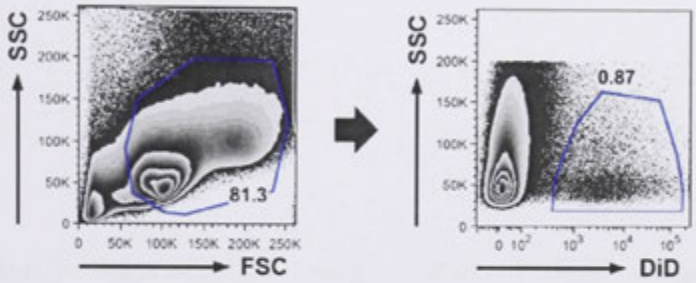


Figure 2-4| Gating scheme to identify transferred splenocytes that were stained with Vybrant DiD labelling solution in the in vivo cytotoxicity assay. A naive C57Bl/6 mouse was i.v. injected with 1×10^7 cells that contained 1:1 ratio of DiD^+ $CFSE^{lo}$ splenocytes that was pulsed with gB-498 peptide and DiD^+ $CFSE^{hi}$ splenocytes that was left unpulsed (as described in Section 2.2.35). Four hours later, splenocytes were prepared from the mouse and were analysed with flow cytometry. To identify the transferred splenocyte population, which was DiD^+, cells were first gated on a SSC × FSC plot, followed by gating on SSC × DiD plot. The percentages of $CFSE^{lo}$ and $CFSE^{hi}$ cells were determined within this population and used to determine the percentage of specific lysis of gB-498-presenting cells (refer to Figure 6-10A and Section 2.2.35). The number in each plot represents the percentage of events of the gated population relative to the population shown in the plot.

Chapter 3 Anti-vaccinia virus $CD8^+$ T cell priming pathways cannot be dissected with systemic Toll-like receptor 9 ligation or selective killing of dendritic cell subsets with cytochrome c

3.1 Introduction

Understanding how $CD8^+$ T cells are primed during immunisation with VACV, especially the highly attenuated strains such as MVA, has practical relevance for designing better VACV-based recombinant vaccines. In addition, the lack of consistency in the literature about priming mechanisms across the VACV strains indicates that further research is needed to dissect priming pathways for anti-viral $CD8^+$ T cells.

Most studies on antigen presentation during VACV immunisation focused on a few model antigens expressed from recombinant WR, such as OVA (Norbury et al., 2001; Norbury et al., 2004; Ma et al., 2009; Xu et al., 2010) and β-galactosidase (Shen et al., 2002; Tewalt et al., 2009). These data were important for establishing how $CD8^+$ T cell responses can be induced during VACV immunisation, but more information is necessary before generalising these findings. More importantly, only one study directly addressed the priming pathway used by MVA and suggested that antigens expressed from MVA are mainly cross presented to induce $CD8^+$ T cell immunity in vivo (Gasteiger et al., 2007). This study mainly focused on a tumor model antigen, Tyr, and OVA expressed as various constructs from recombinant MVA. Clearly, further research with more antigens is required to examine the conclusion of this study.

Here, for the purpose of this thesis, the word 'antigen' is used to describe full-length proteins where the antigenic peptides, which bind onto MHC-I, are processed from. '$CD8^+$ T cell epitopes' is used to describe MHC-bound peptides that are known to be recognised by $CD8^+$ T cells isolated from animals or humans that have been immunised or infected.

Presentation of peptides from native VACV antigens to $CD8^+$ T cells in vivo has only been examined in one study. Basta et al. (2002) immunised mice with a recombinant WR expressing the human cytomegalovirus proteins US2 or US11, which down-regulate MHC-I and prevent direct presentation on the infected cells (Jones et al., 1995; Jones and Sun, 1997). When mice were immunised with these recombinant viruses, $CD8^+$ T cell responses against some VACV $CD8^+$ T cell

epitopes were selectively suppressed while a subset of VACV peptides remained immunogenic (Basta et al., 2002). Further, $CD8^+$ T cells against the latter group of VACV peptides could be cross-primed from the lysates of MHC-I mismatched cells that were infected with WR (Basta et al., 2002). These data suggest that some VACV antigens can be cross presented in vivo. However, this study focused on antigenic peptides fractionated from APCs infected with VACV in vitro with the use of reverse-phase high-performance liquid chromatography, and the identities of the presented peptides were not determined. Moreover, the effects of US2 and US11 on the in vitro expression of $H\text{-}2D^b$ and $H\text{-}2K^b$ molecules contradicted the effects of these proteins on the in vivo presentation of antigens on these two MHC-I alleles (Basta et al., 2002). This further complicates the interpretation. More recently, numerous immunogenic VACV $CD8^+$ T cell epitopes have been mapped in C57Bl/6 mice (Tscharke et al., 2005; Moutaftsi et al., 2006; Yuen et al., 2010). Although it has been shown that responses against some of these mapped epitopes can be elicited via cross presentation from VACV-infected cells (Lev et al., 2009), it remains unknown how $CD8^+$ T cells against most of these epitopes are primed during natural VACV immunisation. Examining the MHC-I presentation pathways of this large panel of VACV $CD8^+$ T cell epitopes in vivo would improve our understanding of $CD8^+$ T cell priming by VACV in general.

Several strategies can be used to examine how individual antigens are presented from a virus. As discussed above, recombinant viruses expressing proteins to down regulate MHC-I presentation pathway can be used in vivo (Basta et al., 2002; Shen et al., 2002). APCs infected with such recombinant viruses will not present peptides via direct presentation and in vivo direct priming will be suppressed. However, cross priming should not be affected as the cross presenting APCs are not infected. Thus, a reduction in the $CD8^+$ T cell response against a given peptide induced by the recombinant viruses, compared to the control virus, indicates the contribution of direct priming. Alternatively, an unaffected immune response suggests a role for cross priming. Several proteins have been used to inhibit the direct presentation pathway in this context, such as the human cytomegalovirus proteins US2 or US11 described above (Basta et al., 2002; Shen et al., 2002), the HSV ICP47 protein which inhibits translocation of peptides via

TAP (Hill et al., 1995; Berger et al., 2000), or the cowpox virus CPXV12 and CPXV203 proteins which inhibit TAP ability to transport peptides and suppress MHC-I export to the cell surface respectively (Byun et al., 2007; Alzhanova et al., 2009; Byun et al., 2009; Gainey et al., 2012; Lin, 2013). For this strategy, it is essential to use inhibitory proteins that can completely block direct presentation because even a highly reduced level of peptides presented on cells infected with the recombinant virus may still allow effective direct priming (Lin, 2013).

A second method involves mutant mouse models which do not express components essential for the antigen presentation pathway. TAP-deficient mice have been used to study how different constructs of OVA and IAV NP antigen expressed from VACV WR are presented in vivo (Norbury et al., 2001). In another study, Xu et al. (2010) generated bone marrow chimeric mice by reconstituting lethally-irradiated wildtype mice with the bone marrow from MHC-I-deficient mice (MHC-I-deficient→wildtype chimera). By immunising these mice with a recombinant WR expressing the MHC-I H-2K^b molecule, they found that direct presentation by bone marrow-derived APCs is essential for priming a CD8$^+$ T cell response against the immunodominant VACV peptide B8-20 (Xu et al., 2010). The importance of a particular cross presentation mechanism can also be examined with transgenic mouse models, such as the cathepsin S-deficient mice, which are defective in the vacuolar cross presentation pathway (Shen et al., 2004).

The two strategies mentioned above require mutant mice and/or recombinant viruses. Recently, two new methods that inhibit cross presentation by DCs in wildtype mice have been reported. The first method requires pre-treatment of mice with CpG (Wilson et al., 2006), a toll-like receptor (TLR)-9 agonist (Hemmi et al., 2000). CpG is internalised by the DEC-205 receptor on the surface of DCs (Lahoud et al., 2012) and is then translocated into lysosomes where it binds to TLR-9 (Takeshita et al., 2001; Ahmad-Nejad et al., 2002). Binding of TLR-9 leads to activation of the myeloid differentiation marker (MyD) 88 adapter molecule which ultimately leads to DC maturation (Häcker et al., 2000; Schnare et al., 2000; Kaisho and Akira, 2001; Kaisho et al., 2001; Hemmi et al., 2003). CpG can be further classified into three different classes (class A, B and C)

depending on the nucleotide sequences (Vollmer et al., 2004). The three classes of CpG have different immmunostimulatory effects and the inhibitory effect of CpG class B on cross presentation is discussed here. Injecting mice i.v. with this class of CpG causes systemic maturation of DCs resulting in their loss of ability to cross present antigens, but direct presentation is apparently not affected (Wilson et al., 2006). It should be noted that the timing of CpG injection dictates the outcome of the treatment. It has been shown that immunisation of mice with soluble antigens together with CpG or with CpG-conjugated soluble antigens leads to cross priming of antigen-specific $CD8^+$ T cells (Sparwasser et al., 2000; Maurer et al., 2002). In this chapter, on the other hand, the reported feature of CpG pre-treatment to suppress cross presentation in vivo was exploited.

Secondly, systemic administration of horse cytc before immunisation selectively triggers apoptosis in cross-presenting $CD8\alpha^+$ DCs and $CD103^+$ DCs and results in the inhibition of cross presentation (Lin et al., 2008; Qiu et al., 2009). This targeted killing is a result of the unique ability of these DCs to translocate extracellular proteins into their cytoplasm (Lin et al., 2008). In this case, any captured cytc available in the cytoplasm binds to the apoptotic protease activating factor-1 to trigger apoptosis (Zou et al., 1997; Lin et al., 2008). Both CpG and cytc methods have been used in studies of cross presentation mechanisms (Farrand et al., 2009; Imai et al., 2011) and of $CD8^+$ T cell priming pathways in various models, including HSV-1 infection (Allan et al., 2003; Wilson et al., 2006), *Plasmodium* infection (Chakravarty et al., 2007; Cockburn et al., 2011), and allograft transplantation (Sutherland et al., 2011).

Importantly, CpG treatment has been applied in two separate studies to VACV WR and MVA. Xu et al (2010) demonstrated that this treatment does not reduce $CD8^+$ T cell responses to a directly presented OVA-257 construct and the native VACV B8-20 peptide expressed from WR. In contrast, Gasteiger et al (2007) showed the opposite when mice were immunised with MVA. In this latter study, CpG treatment suppressed $CD8^+$ T cell responses against all tested peptides expressed from MVA. Based on the reported inhibitory effect of CpG on cross priming, these contradictory results were used as evidence to support the arguments that antigens expressed from WR are directly presented while antigens

from MVA are cross presented for $CD8^+$ T cell priming. However, there has not been a direct comparison of cross and direct priming with these two VACV strains. Further, no single study has used both the CpG and cytc methods to see whether these two methods give similar results for any model system.

In this chapter, the antigen presentation mechanisms for individual native antigens expressed from VACV WR and MVA were investigated. Firstly, an assay for examining cross presentation from VACV-infected cells in vivo was established. Using this assay, several VACV antigens were shown to be cross presented from VACV-infected cells to prime $CD8^+$ T cells. The CpG (class B) and cytc pre-treatments were then used with the aim of dissecting how native antigens from WR and MVA prime $CD8^+$ T cells during VACV immunisation. Contrary to previous reports, results here demonstrate that these two commonly used methods do not separate priming pathways of VACV antigens.

3.2 Results

3.2.1 $CD8^+$ T cell immunity against a panel of 14 VACV peptides is induced during dermal immunisation with VACV WR or MVA

(For the ease of reference, the viruses used in this chapter are listed in Table A-1 in the Appendix)

Most studies on MHC-I presentation of VACV-expressed antigens focused on $CD8^+$ T cell responses induced during systemic immunisations, such as through the i.p. or i.v. routes (Norbury et al., 2001; Gasteiger et al., 2007; Xu et al., 2010). In these cases, VACV spreads to multiple internal organs and secondary lymphoid organs (Buller et al., 1985; Rodriguez et al., 1988; Ramírez et al., 2000; Benning and Hassett, 2004; Naik et al., 2006; Gómez et al., 2007; Lin et al., 2013). However, during vaccination with VACV in humans, dermal immunisation is used as discussed in Section 1.9.1. The $CD8^+$ T cell immune responses induced during a peripheral route of immunisation may be different to the better studied systemic routes. Therefore, we focused on i.d. immunisation with VACV in ear pinnae (Tscharke and Smith, 1999; Lin et al., 2012). Here the virus is restricted to the infected ears and draining LNs even after injection with a high dose (10^6 PFU)

of VACV WR (Tscharke and Smith, 1999; Fischer et al., 2011; Lin et al., 2013), suggesting that this VACV infection is controlled locally.

To examine the $CD8^+$ T cell responses to a large panel of 14 VACV $CD8^+$ T cell epitopes mapped based on i.p. VACV immunisation, C57Bl/6 mice were injected i.d. with VACV strains WR or MVA. Seven days after immunisation at the peak of an $CD8^+$ T cell response (Hassett et al., 2000; Harrington et al., 2002), single cell suspensions of splenocytes from immunised mice were prepared and stimulated with synthetic peptides ex vivo. The responding $CD8^+$ T cells were then identified by ICS of IFN-γ. This assay allows a robust and precise quantification of antigen-specific $CD8^+$ T cells during acute VACV and HSV-1 infections, comparable to peptide/MHC-I tetramer staining and other similar technologies (Flesch et al., 2012).

Fig 3-1A shows representative flow cytometric plots of splenic $CD8^+$ T cells from WR or MVA-immunised mice which produced IFN-γ after stimulation with the peptides indicated. The gate of the IFN-γ^+ population was set from a media-only control for each individual experiment and was applied to all samples of each individual experiment. In some rare scenarios, for example when splenocytes from WR-infected mice were stimulated with the B8-20 peptide as shown in Fig 3-1A, the IFN-γ^- population within the $CD8^+$ population showed a marked increase in the MFI of IFN-γ staining. This situation occurred only when there was a large population of $CD8^+$ T cells responding to a given peptide. A likely explanation is that not all the IFN-γ was blocked from secretion from the large population of responding cells during ex vivo peptide stimulation even in the presence of BFA. Some of the released IFN-γ might become associated with other cells in the culture, and therefore might increase the background IFN-γ staining. To overcome this problem, the IFN-γ^+ gate was adjusted upward slightly in these rare cases to more accurately separate the IFN-γ^+ and IFN-γ^- events, which were clearly two distinct populations.

As seen in Figure 3-1B & C, mice immunised with WR via the i.d. route elicited $CD8^+$ T cell responses against multiple $CD8^+$ T cell epitopes, which can be ranked into a hierarchy based on their immunogenicity. Around 10% of the $CD8^+$ T cells

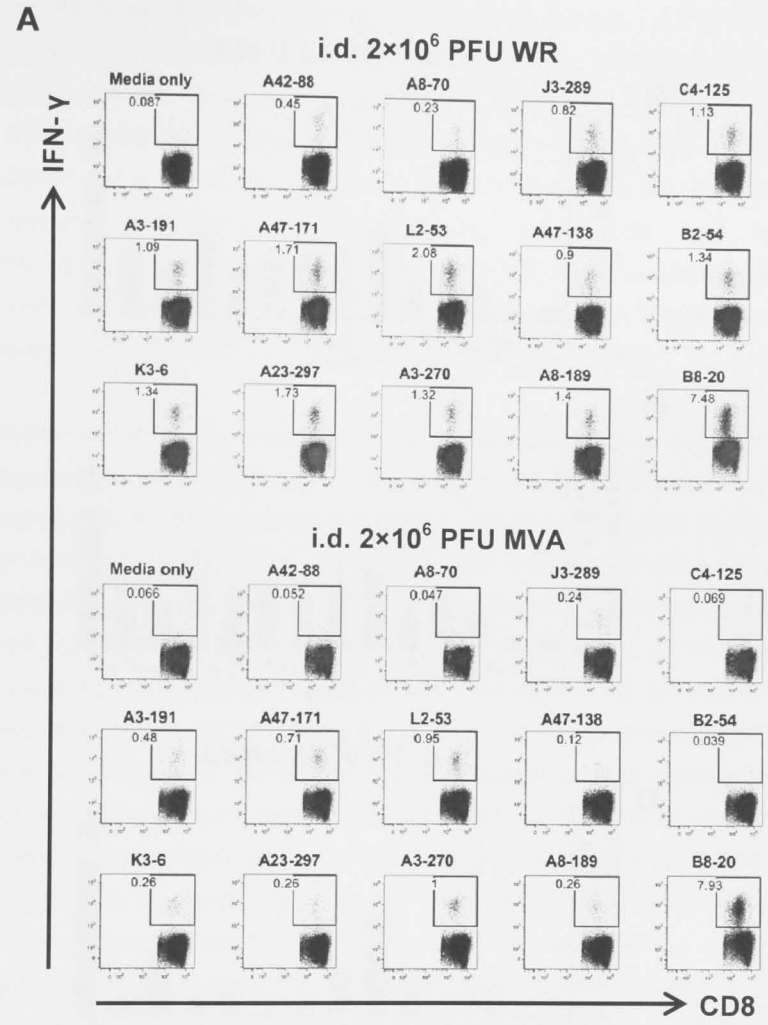
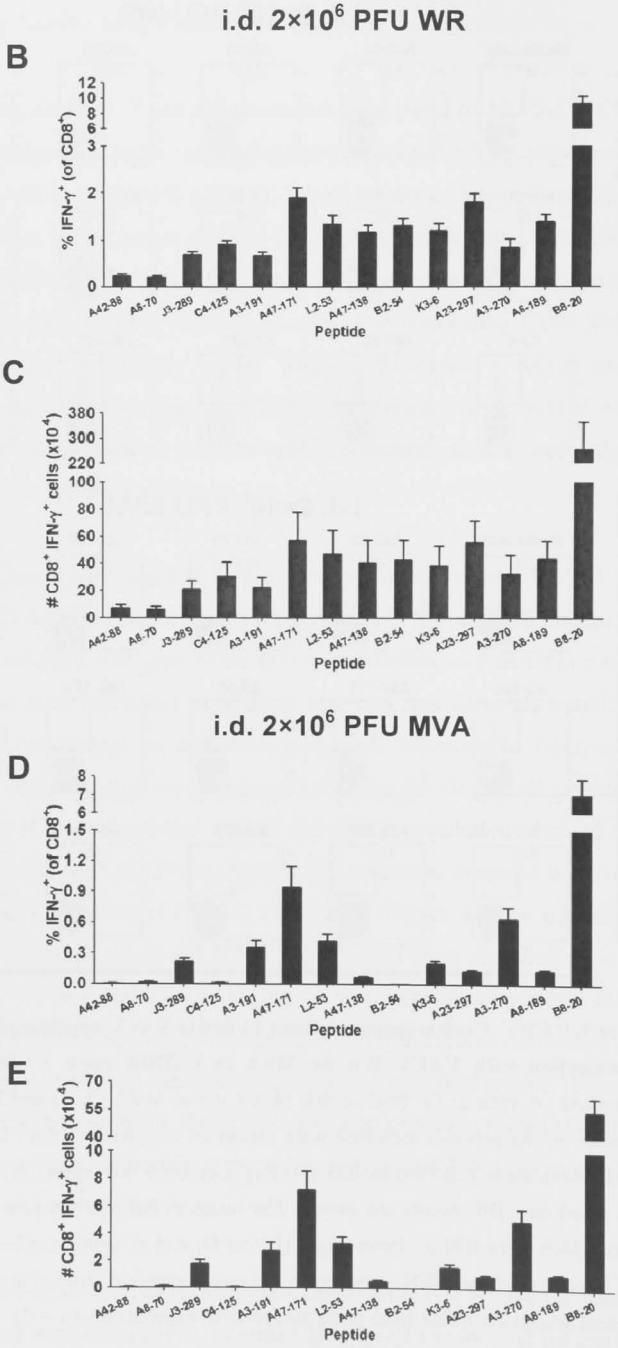


Figure 3-1| CD8$^+$ T cell responses against 14 native VACV peptides elicited after i.d. immunisation with VACV WR or MVA in C57Bl/6 mice. C57Bl/6 mice were immunised i.d. with 2×10^6 PFU of WR (A-C) and or MVA (A, D and E). CD8$^+$ T cell responses to the peptides indicated were measured in spleens 7 days later by ex vivo peptide stimulation, followed by ICS. (A) Representative flow cytometry (IFN-γ × CD8) plots gated on CD8$^+$ events are shown. The numbers indicate the percentage of CD8$^+$ events which were IFN-γ$^+$. Percentages (B and D) and absolute numbers (C and E) of CD8$^+$ T cells producing IFN-γ are shown as mean ± standard error of mean (SEM). Data represent results compiled from three independent experiments (n = 9). Please note that graphs B to E are shown on the following page.


i.d. 2×10^6 PFU WR
B
% IFN-γ+ (of CD8+)
12
10
8
6
3
2
1
0
A42-88
A8-70
J3-289
C4-125
A3-191
A47-171
L2-53
A47-138
B2-54
K3-6
A23-297
A3-270
A8-189
B8-20
Peptide
C
CD8+ IFN-γ+ cells (x10^4)
380
300
220
100
80
60
40
20
0
Peptide
i.d. 2×10^6 PFU MVA
D
% IFN-γ+ (of CD8+)
8
7
6
1.5
1.2
0.9
0.6
0.3
0.0
Peptide
E
CD8+ IFN-γ+ cells (x10^4)
70
55
40
10
8
6
4
2
0
Peptide

were B8-20-specific, which was equivalent to approximately 2.5×10^6 B8-20-specific $CD8^+$ T cells. It was followed by a large group of subdominant VACV peptides, each of which was recognised by around 1-2% of $CD8^+$ T cells (2.0-5.5×10^5 specific $CD8^+$ T cells). This group of peptides included A8-189, A3-270, A23-297, K3-6, B2-54, A47-138, L2-53, A47-171, A3-191, C4-125 and J3-289. Furthermore, about 0.2% of $CD8^+$ T cells, or around 7×10^5 $CD8^+$ T cells, from WR-immunised mice were specific to A8-70 or A42-88. This immunodominance hierarchy of individual native VACV epitopes induced during i.d. immunisation is similar to that induced by i.p. immunisation (Moutaftsi et al., 2006).

Similar to WR, B8-20 remained as the immunodominant peptide during MVA immunisation (Figure 3-1D & E). MVA induced a B8-20-specific $CD8^+$ T cell response which made up around 8% of the total $CD8^+$ T cell population. However, the immunodominance hierarchy of the subdominant peptides induced by MVA is different to that of WR. A47-171 is clearly the second most immunogenic peptide while A3-270 ranked third, recognised by around 1% and 0.8% of $CD8^+$ T cell respectively. The immunogenicity of the rest of the peptides was less than 0.5% each. Notably, the B2-54 and C4-125 peptides are not expressed by MVA (Antoine et al., 1998) and as expected no responses to these peptides were detected. Further, MVA primed much smaller $CD8^+$ T cell responses against the examined peptides than WR, as measured by absolute numbers of responding $CD8^+$ T cells. For example, only 5.5×10^5 $CD8^+$ T cells were found to be specific to the immunodominant B8-20 peptide in MVA-immunised mice, a response approximately 80% smaller than the antigen-specific population induced by WR. This suggests that MVA provides less co-stimulation for $CD8^+$ T cell priming than WR, as has been suggested for other less virulent strains of VACV (Salek-Ardakani et al., 2011a). MVA may also provide a lower antigen dose compared to WR as it does not replicate in vivo. In spite of these differences between the two VACV strains in inducing $CD8^+$ T cell immunity, both are capable of inducing responses against multiple native VACV peptides.

3.2.2 The use of heat treatment to inactivate residual VACV on infected antigen donor cells in an in vivo cross priming assay

After establishing that many peptides from native VACV antigens are immunogenic when mice were immunised with VACV WR or MVA via the i.d. route, we were interested in the presentation pathways involved in priming their responses. Firstly, we examined if $CD8^+$ T cells against any of these peptides can be cross primed. For this purpose, an in vivo cross priming assay was required. Cells deficient in MHC-I or expressing mismatched MHC-I have been used as antigen donor cells in many studies to examine cross priming (Norbury et al., 2004; Shen and Rock, 2004; Wolkers et al., 2004; Basta et al., 2005). In this assay, $CD8^+$ T cells from the immunised mice cannot directly recognise peptides presented on the surface of antigen donor cells. In this thesis, human 293A cells infected with VACV were used as antigen donor cells. These cells express human MHC-I which cannot be recognised by murine $CD8^+$ T cells and they have been used in cross presentation assays in mice in various studies (Norbury et al., 2004; Donohue et al., 2006; Lev et al., 2009).

Because VACV-infected cells were used to induce cross priming in vivo, residual viral particles associated with the infected cells must be completely inactivated to abolish their infectivity. Complete viral inactivation eliminates the possibility of infection of APCs in the host and resulting direct presentation. Several methods have been used to inactivate viruses, including psoralen/UV treatment to crosslink viral DNA (Hearst and Thiry, 1977; Hanson et al., 1978), and heat inactivation to denature viral proteins (Kaplan, 1958; Woodroofe, 1960; Madeley, 1968).

Firstly, the ability of psoralen derivatives, namely TMP and AMT, to inhibit viral replication and viral gene expression were tested. Psoralen and its derivatives covalently attach to pyrimidines on RNA and DNA and form crosslinks between the opposite strands of DNA in the presence of UV irradiation (Musajo et al., 1967; Song and Tapley, 1979). This treatment has been used to inactivate different viruses, including those with large DNA genomes such as VACV and HSV (Hearst and Thiry, 1977; Hanson et al., 1978; Hanson, 1992; Tsung et al., 1996; Fischer et al., 2007). To test their efficiency at inhibiting VACV replication and gene expression, a WR recombinant, namely WR-TK-SIIN (Restifo et al.,

1995), was chosen as the model virus. This recombinant virus expresses the dominant $CD8^+$ T cell epitope in OVA, OVA-257, as a cytosolic minigene (MSIINFEKL) under the control of the VACV p7.5 early/late promoter from the VACV TK locus. Because of the short length of the minigene (27 bp long), suppression of its expression from VACV with psoralen requires extended UV exposure compared to the full-length OVA antigen (Fischer et al., 2007). More importantly, the OVA-257 minigene expressed from WR can only be directly presented but not cross presented (Serna et al., 2003; Norbury et al., 2004). Therefore, a $CD8^+$ T cell response induced against the OVA-257 minigene from WR-TK-SIIN-infected cells that were treated with an inactivation method would imply an incomplete inactivation of the virus. With this virus, we could stringently examine VACV inactivation.

TMP was first examined. WR-TK-SIIN was treated with 10 μg/ml of TMP and irradiated with UV light at 365 nm. This concentration of TMP has been used in previous studies to inactivate VACV (Norbury et al., 2004; Fischer et al., 2007). After irradiation with UV for 20 min, the replicative ability of the TMP-treated virus was reduced to below the detection limit of the standard plaque assay (Figure 3-2A). To examine if this treatment also inhibited VACV gene expression and antigen presentation, we took advantage of 293-K^b cells and 25D1.16 antibody. 293-K^b cells are human 293A cells stably expressing mouse MHC-I H-$2K^b$ (Tscharke et al., 2005), the restricting MHC-I of OVA-257 (Carbone and Bevan, 1989; Falk et al., 1991b; Rötzschke et al., 1991). 25D1.16 antibody recognises the OVA-257/H-$2K^b$ complex (Porgador et al., 1997), and so this reagent allows a direct measurement of MHC-I presentation of OVA-257. Here the surface expression level of OVA-257/H-$2K^b$ was measured with 25D1.16 antibody on 293-K^b cells that had been incubated with the treated viruses for six hours. The results on Figure 3-2B show that TMP treatment of WR-TK-SIIN followed by up to an hour of UV irradiation only moderately reduced surface OVA-257/H-$2K^b$ expression on the inoculated 293-K^b cells, suggesting that TMP/UV treatment did not completely inactivate VACV.

Another psoralen derivative, AMT, was also examined. It has been reported that AMT can completely inhibit VACV viral gene transcription (Tsung et al., 1996).

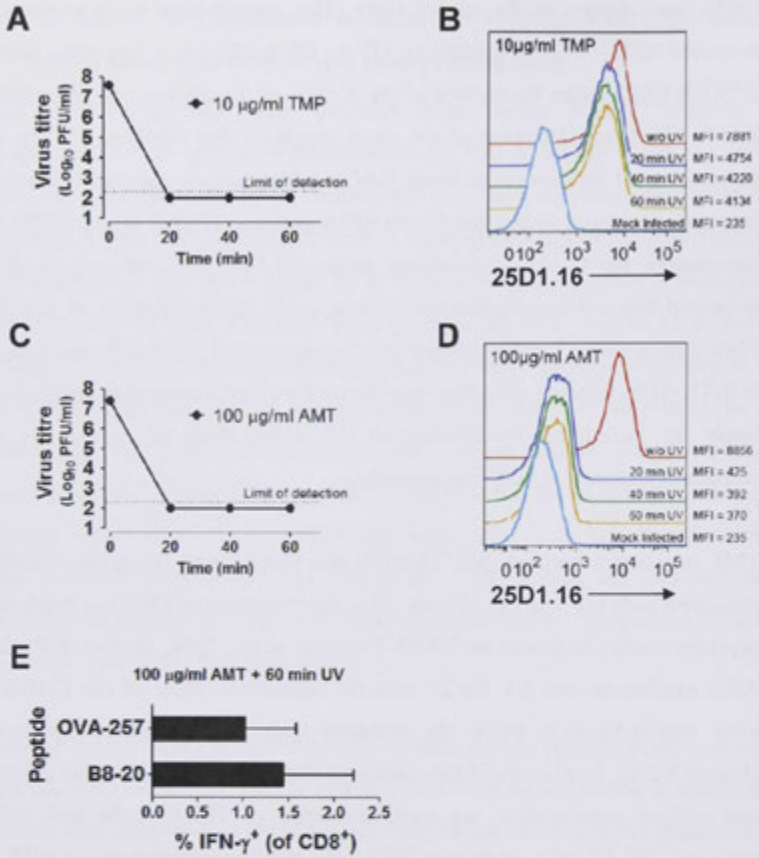


Figure 3-2| Psoralen/UV treatment does not completely stop the OVA-257 minigene expression from residual VACV used during infection of cells in vitro. (A-D) 1×10^8 PFU of WR-TK-SIIN was treated with 10 µg/ml TMP (A and B) or 100 µg/ml AMT (C and D) in PBS in a final volume of 500 µl. The solutions were irradiated with UV-A light (365 nm). At times indicated, 100 µl of virus solution was taken for further experiments. (A and C) Residual viral infectivity was determined by standard plaque assay. (B and D) OVA-257 minigene expression and MHC-I presentation was determined by infecting 293A-K^b cells with the treated virus at an m.o.i. of 5, followed by 25D1.16 antibody staining 6 h later. (A-D) Results represent data from a single experiment. (E) 293A cells were infected with WR-TK-SIIN at an m.o.i. of 5 for 6 h. These infected cells were then treated with 100 µg/ml AMT and 60 min UV irradiation (4×10^7 cells in 2 ml). A group of three mice were then immunised i.p. with 1×10^7 infected AMT/UV-treated cells. $CD8^+$ T cell responses to OVA-257 and VACV B8-20 were measured in spleens 7 days later via ICS. The percentages of $CD8^+$ T cells producing IFN-γ are shown as mean ± SEM (n = 3; from a single experiment).

In comparison to TMP used above, a higher concentration of AMT was applied here to ensure better viral inhibition. Similar to TMP, WR-TK-SIIN treated with 100 μg/ml of TMP and irradiated with UV light at 365 nm for 20 min abolished the viral replication ability as detected by standard plaque assay (Figure 3-2C). Contrary to TMP, as little as 20 min of UV irradiation greatly reduced the level of OVA-257/H-2K^b presentation on the surface of 293-K^b cells inoculated with AMT-treated virus (Figure 3-2D). However, a closer examination revealed that the inhibition of the OVA-257 minigene presentation from AMT-treated WR-TK-SIIN might not be complete even after an hour of UV irradiation. This was suggested by the slightly higher MFI of the 25D-1.16 antibody staining on the cells inoculated with the AMT-treated virus, compared to the mock-infected cells (Figure 3-2D). To empirically demonstrate whether AMT/UV treatment could be used to completely inactivate residual virus on infected cells in a cross priming assay, 293A cells that had been infected with WR-TK-SIIN for 6 h were treated with 100 μg/ml AMT and irradiated with UV for 60 min. Mice were then immunised with these treated cells. Seven days after immunisation, OVA-257-specific CD8$^+$ T cell immunity was induced by the AMT/UV-treated WR-TK-SIIN-infected cells (Figure 3-2E). This result demonstrates that AMT/UV treatment did not completely inhibit viral gene expression from residual VACV associated with the infected cells.

The usefulness of heat inactivation was then investigated. Heating VACV to 55-65°C has been used to suppress its infectivity (Kaplan, 1958; Woodroofe, 1960; Madeley, 1968). Unlike psoralen/UV treatment which targets VACV DNA genome, heat treatment denatures VACV viral proteins and ablates their functions even at 45°C (Berns et al., 1969). Here WR-TK-SIIN was heat-treated at 60°C for 60 min. This treatment inhibited the replicative ability of the virus to below the detection limit (Figure 3-3A). Heat treatment also completely inhibited growth of wildtype WR and other VACV recombinants as determined by standard plaque assay (data not shown). Furthermore, 293-K^b cells inoculated with the heat-inactivated WR-TK-SIIN did not present the OVA-257 minigene as measured by surface OVA-257/H-2K^b expression with 25D1.16 antibody labelling (Figure 3-3B). An independent experiment with WR recombinant expressing an ER-targeted OVA-257 minigene confirmed this finding (data not shown). Finally, mice

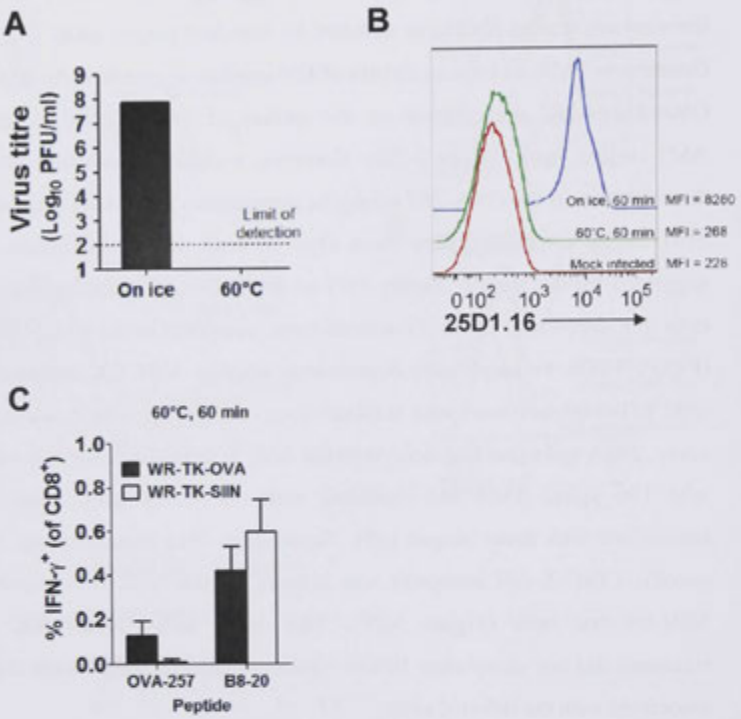


Figure 3-3| Heating VACV-infected cells to 60°C for 60 min completely inhibits the OVA-257 minigene expression from residual VACV. (A) 8×10^7 PFU of WR-TK-SIIN in 400 µl of PBS was either incubated on ice or at 60°C for 60 min. The replicative ability of the treated virus was determined by standard plaque assay. (B) 293-K^b cells mock-inoculated or inoculated with WR-TK-SIIN that was either put on ice or heat-treated at 60°C for 60 min at an m.o.i. of 5 for 6 h. OVA-257/H-$2K^b$ complex expression on cell surface was measured with 25D1.16 antibody. (C) 293A cells were infected with either WR-TK-OVA or WR-TK-SIIN at an m.o.i. of 5 for 6 hours. Cells were then heat treated at 60°C for 60 min in PBS in a volume of 500 µl (4×10^7 cells). Groups of three mice were then immunised i.p. with 1×10^7 infected heat-treated cells. $CD8^+$ T cell responses to the OVA-257 and VACV B8-20 peptides were measured in spleens 7 days later via ICS. The percentage of $CD8^+$ T cells producing IFN-γ are shown as means ± SEM. (A and B) Results represent data from one experiment. (C) Results shown are compiled from two independent experiments (WR-TK-OVA: n = 5; WR-TK-SIIN: n = 6).

immunised with 293A cells infected with WR-TK-SIIN followed by heat inactivation did not show any OVA-257-specific $CD8^+$ T cell response (Figure 3-3C). This was not due to the inability of the infected heat-treated cells to provide antigens for cross presentation because a B8-20-specific response was detected in these mice. Also, cells infected with WR-TK-OVA, a WR recombinant expressing full-length OVA that is known to be cross presented from VACV-infected cells (Serna et al., 2003; Norbury et al., 2004; Lev et al., 2009), elicited a measurable OVA-257-specific $CD8^+$ T cells response (Figure 3-3C). The failure of the OVA-257 minigene, but not full-length OVA, to induce an OVA-257-specific $CD8^+$ T cell response from the VACV-infected cells indicates that the minigene is completely inactivated from residual virus on the infected cells after heat treatment. Therefore, this method was chosen to inactivate the VACV-infected cells used for our cross priming assay. To ensure that no active virus was associated with the infected cells for our cross priming experiments after heat treatment, the infected cells were routinely checked for their lack of replicative ability and their inability to infect fresh APCs for direct presentation in vitro (data not shown).

3.2.3 Some VACV antigens can be cross presented from VACV-infected cells to prime $CD8^+$ T cells

Next, we examined whether any of the $CD8^+$ T cell responses against the native VACV peptides tested in Figure 3-1 could be cross primed using the newly established cross priming assay. 293A cells were infected with WR or MVA for 6 h, followed by heat inactivation at 60°C for 60 min. Six hours of infection should allow expression of all VACV antigens tested in this thesis (Assarsson et al., 2008; Yang et al., 2010). Mice were then immunised i.p. with 1×10^7 of these heat-treated VACV-infected cells. Seven days after immunisation, responses against several of the VACV antigens were detected directly ex vivo (Figure 3-4). Even under conditions where only cross presentation was allowed, B8-20 remained the most immunogenic peptide for WR and MVA, representing 2 and 3% of $CD8^+$ T cell response respectively (Figure 3-4A and C). For WR, the next most immunogenic peptide was A3-270, which induced a response of a similar size to B8-20. Several other native VACV peptides from WR were also immunogenic in

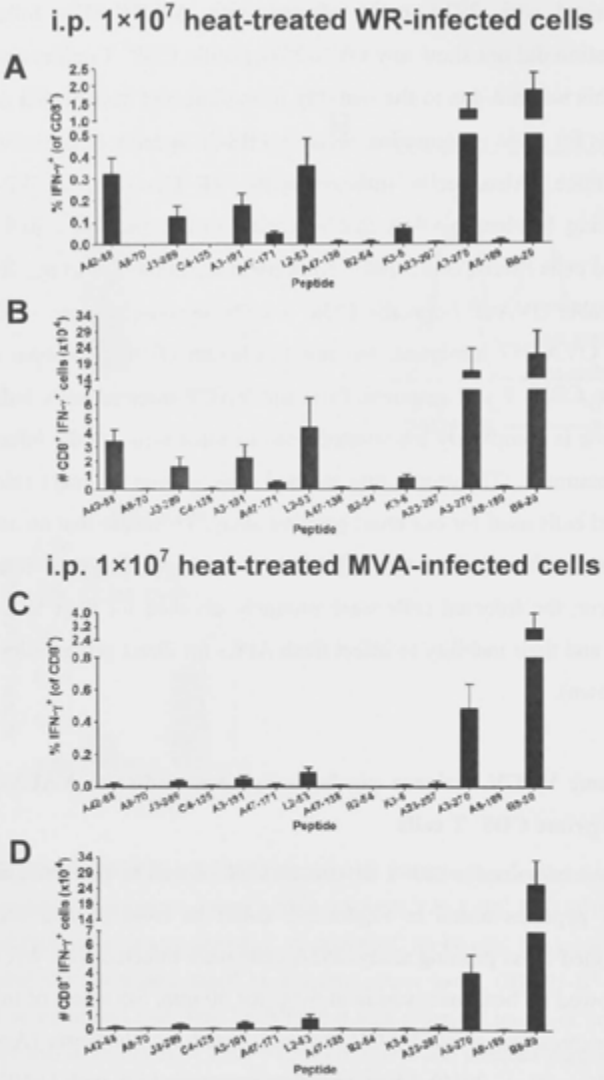


Figure 3-4| CD8$^+$ T cell responses against some VACV antigens can be elicited in mice by cross presentation from VACV-infected, heat-treated cells. 293A cells were infected with WR (A and B) or MVA (C and D) for 6 h at an m.o.i. of 5 at 37°C, followed by heat treatment at 60°C for 60 min to inactivate the residual virus. Mice were immunised i.p. with 1×10^7 of these infected, heat-treated cells. CD8$^+$ T cell responses were measured in spleens 7 days later, after ex vivo peptide stimulation followed by ICS. Data are shown as percentages (A and C) or absolute numbers (B and D) of CD8$^+$ T cells against the indicated peptides (mean ± SEM). Data represent results compiled from two independent experiments (n = 6).

this context, including A42-88, L2-53, J3-289 and A3-191, each of which was recognised by 0.1-0.3% of $CD8^+$ T cells (Figure 3-4A). A similar group of VACV antigens were shown to be cross presented in a previous study in which mice were immunised with 293A cells infected with WR-TK-OVA followed by irradiation with 254 nm UV light (Lev et al., 2009). This suggests that heat treatment is comparable to the germicidal UV treatment used to inactivate residual virus on antigen donor cells. In mice immunised with MVA-infected cells, A3-270 was also the second most immunogenic peptide, similar to that observed in mice immunised with WR-infected cell (Figure 3-4C and D). However, the immunogenicity of the rest of the peptides induced via cross priming was much lower from MVA-infected cells, compared with WR-infected cells. This will be discussed in Section 3.3 (p. 175).

Dermal immunisation with VACV-infected cells was then examined to study if it would produce comparable results. Similar to the result shown in Fig 3-3C in which mice were immunised with WR-TK-SIIN-infected cells via the i.p. route, the OVA-257 minigene did not cross prime an OVA-specific $CD8^+$ T cell response after i.d. immunisation with 2×10^6 WR-TK-SIIN-infected cells (Figure 3-5). This indicates that the OVA-257 minigene was also not cross presented from infected cells during dermal immunisation. The responses against native VACV peptides were then studied. Seven days after an i.d. immunisation with 2×10^6 cells infected with VACV WR or MVA, the overall immunogenicity hierarchies were similar to those of the i.p. route (Figure 3-6, compared with Figure 3-4). It should be noted that the B8-20-specific response induced by MVA-infected cells was significantly higher than that of WR-infected cells ($p < 0.05$ when percentages or total numbers of B8-20-specific $CD8^+$ T cells were compared). This was not observed for other VACV peptides ($p > 0.05$). This might be due to a difference between the amounts of the B8 antigen expressed within cells infected with WR or MVA. In addition, we noticed that the size of the $CD8^+$ T cell immune response, measured as absolute numbers of peptide-specific $CD8^+$ T cells, induced from WR-infected cells seemed to be lower when the i.d. injection route was used, compared with i.p. immunisation (refer to Figure 3-4). However, this is likely to be a dosage effect, not a route-specific effect. As shown in Figure 3-7 below, i.p. immunisation of mice (treated with PBS) with 1×10^6 cells induced a similar

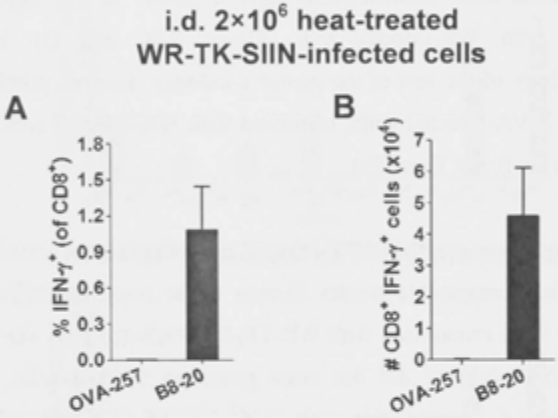


Figure 3-5| Dermal immunisation with WR-TK-SIIN-infected does not cross prime CD8$^+$ T cells against the OVA-257 minigene. 293A cells were infected with WR-TK-SIIN for 6 h at an m.o.i. of 5 at 37°C, followed by heat inactivation at 60°C for 60 min. Mice were immunised i.d. with 2×10^6 of these infected, heat-treated cells. CD8$^+$ T cell responses in the spleens were analysed 7 days later, after ex vivo peptide stimulation and ICS. Data are shown as percentages (A) or absolute numbers (B) of CD8$^+$ T cells responding to peptides indicated (mean ± SEM; n = 3). This experiment was repeated but the repeated experiments were designed to compare the cross priming ability of cells infected with WR-TK-OVA and WR-TK-SIIN. The results are shown in Figure 4-12.

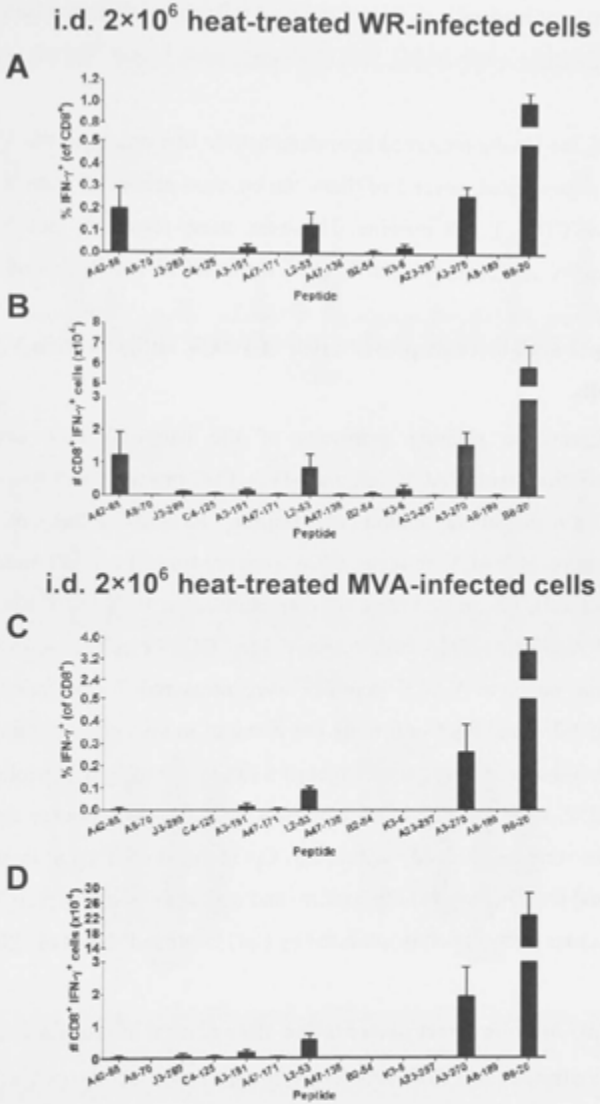


Figure 3-6| Similar to i.p. immunisation, dermal immunisation with VACV-infected can cross prime CD8$^+$ T cell immunity against some VACV antigens. Mice were immunised i.d. with 2×10^6 293A cells, previously infected with WR (A and B) or MVA (C and D) for 6 hours at an m.o.i. of 5 at 37°C, followed by heat inactivation as described in Figure 3-4. CD8$^+$ T cell responses were measured in spleens 7 days later with ex vivo peptide stimulation and ICS. Data are shown as percentages (A and C) or absolute numbers (B and D) of CD8$^+$ T cells against the indicated peptides. Data (mean ± SEM) represent results compiled from two independent experiments (n = 6).

magnitude of responses as those produced following i.d. immunisation with 2×10^6 infected cells.

Overall, the results presented here demonstrate that out of all the VACV $CD8^+$ T cell epitopes tested, several of them can be cross presented from VACV-infected cells for $CD8^+$ T cell priming. However, these results do not predict if these peptides are also cross presented during the standard VACV immunisation.

3.2.4 CpG inhibits cross presentation of VACV antigens from VACV-infected cells

To dissect the priming pathways of the native VACV antigens during immunisations with VACV WR and MVA, CpG pre-treatment was chosen, on the basis that it should only inhibit cross priming. To establish that CpG inhibits cross presentation of VACV antigens, mice were pre-treated with 20 nmol CpG or PBS (as a control). The treated mice were immunised i.p. with 1×10^6 heat-treated, WR-TK-OVA-infected 293A cells one day later. $CD8^+$ T cell responses to OVA and the large panel of VACV peptides were measured 7 days post immunisation (Figure 3-7 A and B). Confirming our previous in vivo cross priming experiments (Figure 3-4 and 3-6), responses against a similar set of VACV peptides, as well as OVA-257, were induced in control mice. In contrast, responses against all these peptides were significantly reduced in CpG-treated mice by at least 80% (Figure 3-7C and D). The results here confirm and extend the range of VACV peptides for which cross presentation is inhibited by CpG treatment (Xu et al., 2010).

3.2.5 CpG inhibits direct presentation after dermal immunisation with VACV

After confirming that CpG treatment inhibited cross priming of VACV antigens in vivo, this method was applied to dissect the presentation pathway of VACV antigens after VACV immunisation via the i.d. route. CpG- and PBS-treated mice were i.d. immunised with 2×10^6 PFU of WR or MVA. Seven days later, $CD8^+$ T cell responses were measured. For MVA, $CD8^+$ T cell responses against B8-20, L2-53 and J3-289 were significantly inhibited by CpG treatment, irrespective of the percentages or absolute numbers of responding $CD8^+$ T cells (left of Figure 3-8C and D). Percentages of $CD8^+$ T cell recognising A3-270 and A47-138 were

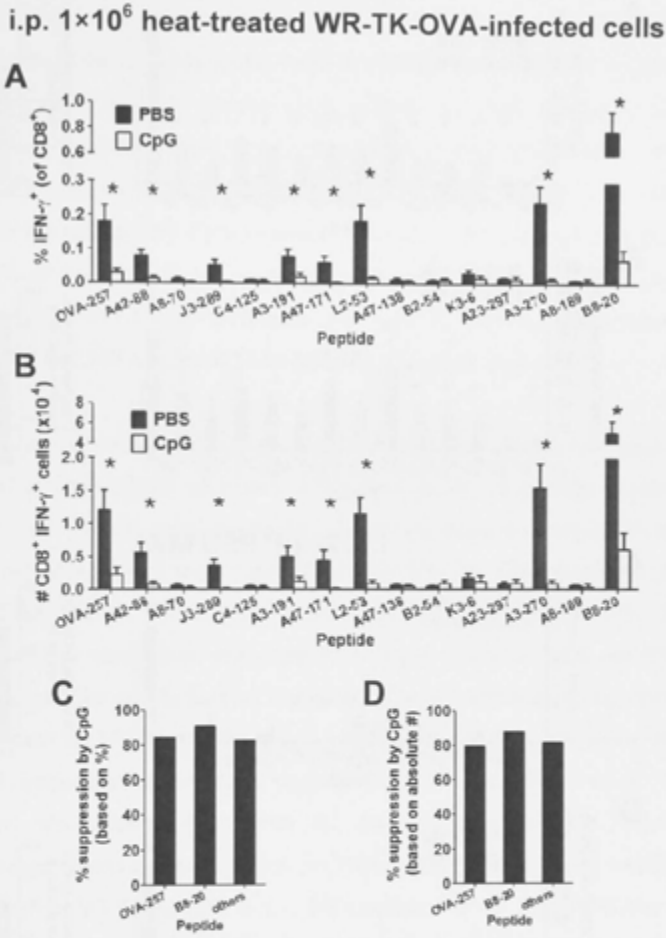


Figure 3-7| CpG treatment inhibits cross presentation. Mice were pre-treated with 10 nM CpG or PBS (control). These mice were then immunised i.p. with 1×10^6 WR-TK-OVA-infected and heat inactivated 293A cells one day later. $CD8^+$ T cell responses to the peptides shown were measured in spleens 7 days post immunisation. The percentages (A) and absolute numbers (B) of responding $CD8^+$ T cells for peptides indicated are shown as mean ± SEM. (C and D) Extent to which responses to OVA-257, B8-20, and the sum of 13 other VACV epitopes (others) were suppressed in CpG compared with control treated mice are shown, based on data in (A) and (B) respectively. * denotes statistical significance ($p < 0.05$). Data are compiled from 3 independent experiments (n = 12).

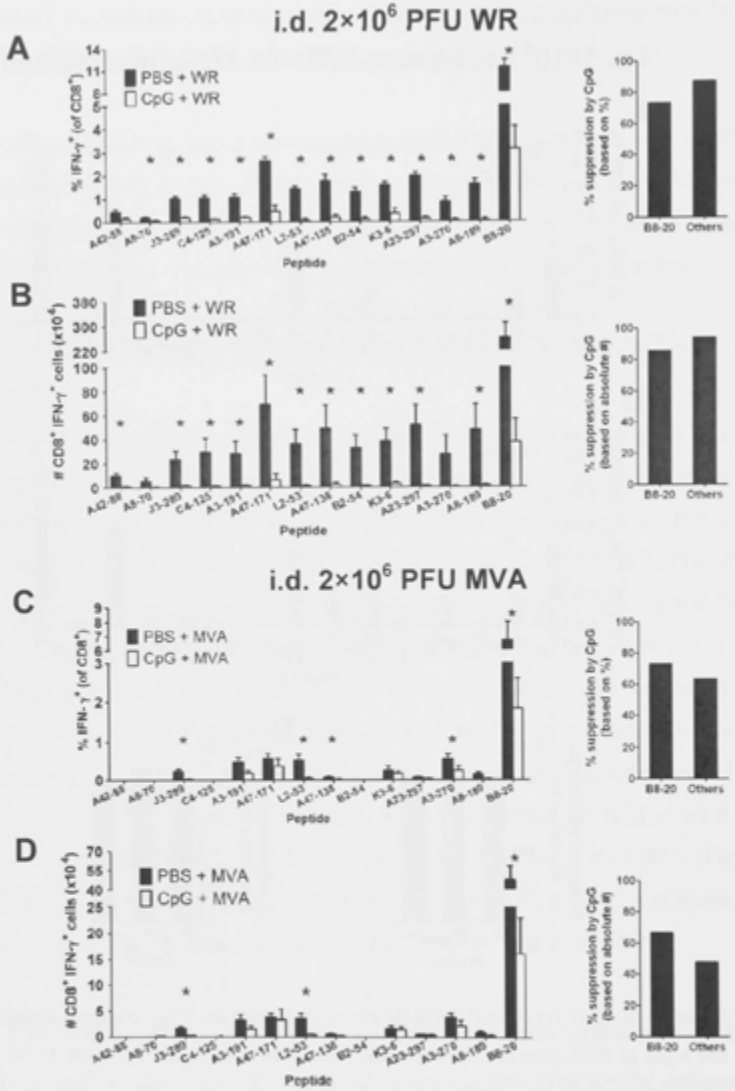


Figure 3-8| CpG treatment suppresses anti-VACV CD8$^+$ T cell immunity after i.d. immunisation. CpG- or PBS-treated mice were immunised i.d. with 2×10^6 PFU VACV strains WR (A and B) or MVA (C and D) and CD8$^+$ T cell responses to the peptides shown were measured in spleens 7 days later. Percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells that made IFN-γ after short stimulation with peptides indicated are shown as mean ± SEM on left. Extent to which responses to B8-20 or the sum of other epitopes (others) were suppressed in CpG compared with control mice based on percentages or absolute numbers are shown on right. Data are compiled from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

significantly reduced after CpG treatment. However, the decreases were not reflected by the absolute numbers of $CD8^+$ T cells that recognised these two peptides, due to the variation in the numbers of $CD8^+$ T cells induced in individual mice. Responses against A3-191 were reduced by CpG treatment, but the difference was not significant when compared with mock-treated mice. We also noticed that there were few exceptions in which responses against K3-6 and A47-171 were not affected by CpG treatment. Overall, CpG treatment suppressed at least 50% of the $CD8^+$ T cell responses induced against native VACV peptides expressed from MVA (right of Figure 3-8C and D). This is in agreement with suggestions that MVA relies on cross priming (Gasteiger et al., 2007).

However, unexpected results were found when $CD8^+$ T cell responses were measured in CpG-treated mice after WR immunisation. In contrast to published data (Xu et al., 2010), priming of $CD8^+$ T cell responses to nearly all examined VACV peptides of WR were significantly inhibited by CpG treatment (left of Figure 3-8A and B). Inhibition by CpG treatment was more than 70% for all epitopes and in some cases more than 80% (right of Figure 3-8A and B). One could interpret the results here as suggesting that cross priming is the dominant pathway both for MVA and WR after i.d. immunisation. This conclusion for WR after i.d. immunisation has been suggested by one study (Shen et al., 2002). However, observations from intravital microscopy using this route and subcutaneous immunisation, another peripheral route, favour direct priming for WR, as well as MVA (Norbury et al., 2002; Hickman et al., 2008; Hickman et al., 2011b; Kastenmüller et al., 2013). It is possible that CpG treatment may influence other functions of the immune system, in addition to its inhibitory effect on cross priming. This could complicate the interpretation of the results here.

To test directly whether CpG treatment genuinely dissects direct and cross priming after i.d. immunisation, WR-TK-SIIN was used again. As shown in Figure 3-5 previously, the OVA-257 minigene expressed from this virus does not cross prime $CD8^+$ T cells from WR-TK-SIIN-infected cells via i.d. immunisation. When PBS-treated mice were i.d. immunised with 2×10^6 PFU of WR-TK-SIIN, nearly 2% of splenic $CD8^+$ T cells recognised the OVA-257 peptide seven days post immunisation (left of Figure 3-9A), which was similar to the response

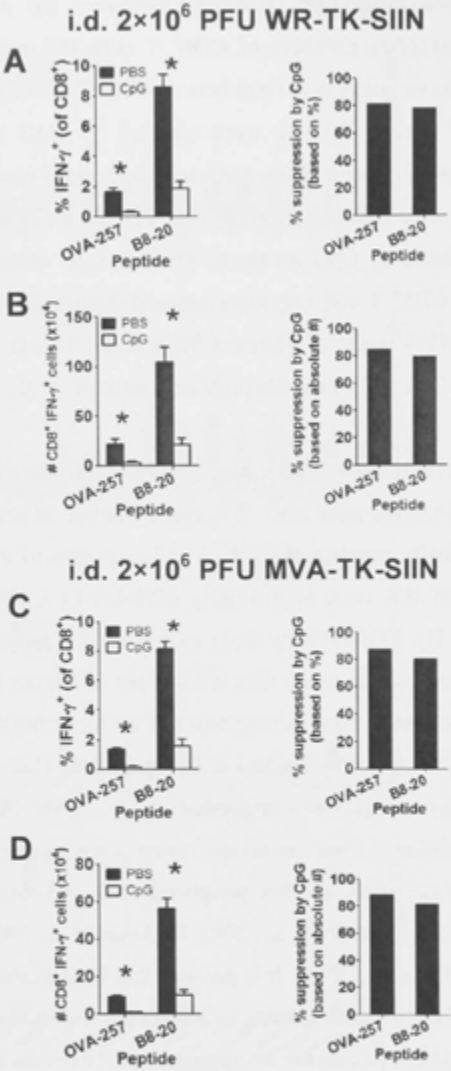


Figure 3-9| CpG treatment suppresses direct priming after i.d. immunisation. CpG- or PBS-treated mice were immunised i.d. with 2×10^6 PFU of WR-TK-SIIN (A and B) or MVA-TK-SIIN (C and D). $CD8^+$ T cell responses to OVA and B8-20 were measured by ex vivo peptide stimulation and ICS 7 days later. Results are shown as percentage of $CD8^+$ T cells (left of A and C) or absolute numbers of $CD8^+$ T cells (left of B and D) producing IFN-γ as mean ± SEM. Graphs on the right show the extent to which responses to OVA-257 and B8-20 were suppressed in CpG compared with control treated mice, based on data on the left. Data are compiled from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

induced by this recombinant virus after i.p. immunisation (see Figure 3-10 and Norbury et al., 2004). This suggests that direct priming is efficient after i.d. immunisation with VACV WR. Moreover, the $CD8^+$ T cell response against this directly-presented OVA-257 construct was inhibited by 80% in mice pre-treated with CpG (right of Figure 3-9A and B). This is similar to the inhibition detected for the immunodominant VACV B8-20 epitope (Figure 3-9A and B). To extend this finding to MVA, a new MVA recombinant which expressed the cytosolic OVA-257 minigene under the VACV p7.5 promoter from the VACV TK insertion site was generated. This virus was specifically made to match WR-TK-SIIN and was named MVA-TK-SIIN. An OVA-257-specific $CD8^+$ T cell response of around 1.5% of $CD8^+$ T cells was induced when mice were immunised with MVA-TK-SIIN via the i.d. route, similar to WR-TK-SIIN (left of Figure 3-9C). This suggests efficient direct priming by MVA. However, the OVA-257-specific response induced by this MVA recombinant was again inhibited by more than 80% in mice treated with CpG, when the response was measured as percentages or absolute numbers of responding $CD8^+$ T cells (right Figure 3-9C and D). Results here indicate that CpG treatment reduces $CD8^+$ T cell responses against antigens expressed from WR and MVA after i.d. immunisation, even if the antigens are exclusively targeted for direct presentation and cannot be cross presented.

3.2.6 The effect of CpG treatment on $CD8^+$ T cell priming depends on antigen, immunisation route and virus replication

To determine whether the results above were specific to i.d. immunisation, the effect of CpG on $CD8^+$ T cell responses induced after systemic i.p. immunisation with WR-TK-SIIN and MVA-TK-SIIN was studied. This also allowed a direct comparison with the published work with CpG, which mainly use systemic routes for immunisation. Unlike the i.d. route, CpG treatment did not reduce OVA-257- and B8-20-specific responses after i.p. injection with WR-TK-SIIN when measured as the percentages of responding $CD8^+$ T cells (Figure 3-10A left). Surprisingly, CpG significantly lowered the responses against most of the other VACV peptides by 60%, suggesting that the effect of this treatment was epitope-specific (Figure 3-10A). When the absolute numbers of responding $CD8^+$ T cells were measured, we found that the size of responses against OVA-257 and B8-20

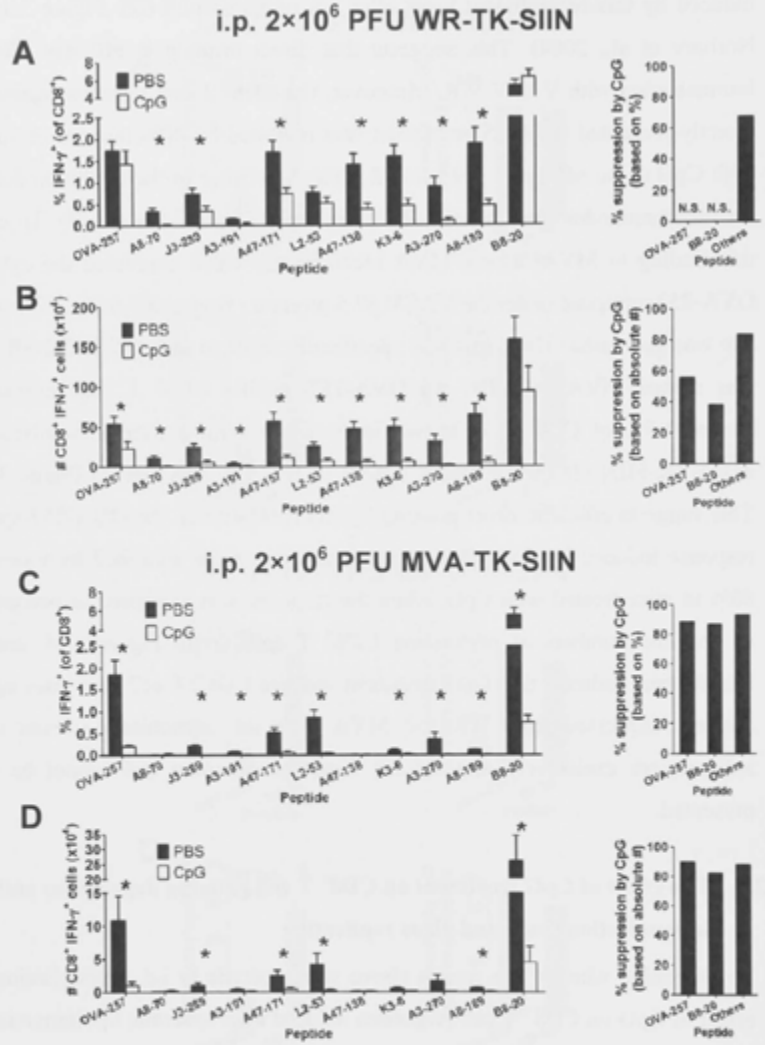


Figure 3-10| CpG treatment shows inconsistent influences on CD8$^+$ T cell responses induced during MVA and WR immunisation via the i.p. route. CpG-treated or control mice (PBS) were immunised i.p. with 2×10^6 PFU of WR-TK-SIIN (A and B) or MVA-TK-SIIN (C, D). CD8$^+$ T cell responses to the peptides shown were measured in spleens 7 days later. Percentages (A, C) and absolute numbers (B, D) of responding CD8$^+$ T cells are shown on graphs on the left as mean ± SEM. Extent to which responses to OVA-257, B8-20, or the sum of other VACV peptides (others) were suppressed in CpG compared with control treated mice after immunisations are shown on graphs on right, based on the data on left. N.S.: not significant. * denotes statistical significance ($p < 0.05$). Results are compiled from three independent experiments (n = 9).

were also reduced by CpG (Figure 3-10B). This implies that CpG might repress the overall magnitude of the anti-WR $CD8^+$ T cell response. However, this phenomenon was not observed by Xu et al. (2010). After i.p. immunisation with MVA-TK-SIIN, CpG treatment significantly inhibited responses to all peptides examined, including B8-20 and the directly-presented OVA-257 minigene, by nearly 90% (Figure 3-10 C and D). Overall, these results confirm published data for WR (Xu et al., 2010) and MVA (Gasteiger et al., 2007) when a systemic immunisation route is used. However, results from the additional VACV peptides for WR and the directly-presented OVA-257 minigene for MVA demonstrate that CpG treatment could not be used to analyse antigen presentation pathways.

The reasons responsible for the differential effect of CpG treatment on $CD8^+$ T cell priming between WR and MVA after i.p. immunisation were examined. While the MVA genome contains several large deletions and multiple smaller mutation regions compared to that of WR (Antoine et al., 1998), the most obvious phenotypic differences between WR and MVA is the inability of MVA to replicate in vivo (Ramírez et al., 2000; Stittelaar et al., 2001; Wyatt et al., 2004). To examine the relationship between VACV replication and the effect of CpG treatment, a new VACV inactivation method was developed to abolish viral replication ability, but allow early virus gene expression. A method based on the TMP/UV treatment of VACV tested in Section 3.2.2 was used. This treatment has been shown to inhibit replication of the treated WR-TK-SIIN while a relatively normal OVA-257/H-$2K^b$ presentation on cells infected with the treated WR-TK-SIIN was detected (refer to Figure 3-2). The amount of TMP and UV exposure time was re-titrated such that it only effectively stops viral replication but does not affect the antigen expression level from WR. Here a WR recombinant, namely WR-NP-S-GFP, was used. This virus expresses a recombinant antigen, namely NP-S-GFP. This antigen consists of the NP antigen from IAV strain PR8, with its carboxyl-terminus fused to the OVA-257 peptide (SIINFEKL) and a GFP (Norbury et al., 2002; Princiotta et al., 2003). This protein is 752 amino acid residues in length, which is longer than 95% of the native VACV proteins. This antigen allowed us to examine how the TMP/UV treatment would affect the expression level of a relatively long antigen expressed from VACV. WR-NP-S-GFP was mixed with 1 μg/ml of TMP and exposed to UV for various lengths of

time. Samples were tested for replicative ability by standard plaque assay and the expression level of NP-S-GFP by measuring GFP fluorescence level in 293A cells inoculated with the treated virus. From the results shown in Figure 3-11A, the TMP-treated virus lost its ability to form plaques after 3 min of UV irradiation. In addition, more than 95% of cells inoculated with WR-NP-S-GFP that had been treated with 1 μg/ml TMP and 3 min UV irradiation expressed GFP (Figure 3-11B). Although the GFP expression level decreased after the treatment, it was still much higher than the mock infected cells (Figure 3-11C). This suggests that long proteins encoded by VACV can be expressed relatively well after the TMP/UV treatment. Thus, treating virus with 1 μg/ml TMP followed by 3 min UV exposure inhibits VACV replication but still allows early viral gene expression.

To test whether replication ability plays a role in determining the outcome of CpG experiments, WR-TK-SIIN was treated with the TMP/UV method as stated above and was injected i.p. into CpG- or PBS-treated mice. The inability of the treated virus to replicate was confirmed by standard plaque assay (data not shown). Seven days after immunisation with the non-replicating WR-TK-SIIN, $CD8^+$ T cell responses against the examined peptides, including OVA-257 and B8-20, were reduced by around 70-90% in CpG-treated mice (Figure 3-12). These results closely resembled the findings using MVA-TK-SIIN (compare to Figure 3-10C and D). Because the response against the directly-presented OVA-257 minigene was suppressed when WR-TK-SIIN was rendered non-replicative, it clearly demonstrates that virus replication, but not priming pathway, influences the outcome of CpG treatment.

3.2.7 CpG treatment reduces virus loads, but this does not explain its impact on $CD8^+$ T cell priming

It remained puzzling why CpG affected priming of $CD8^+$ T cell responses against B8-20 and the OVA-257 minigene more strongly after WR immunisation via i.d. than i.p. injection. As shown in the previous section, the outcome of CpG treatment was sensitive to VACV replication after i.p. immunisation. It is possible that the amounts of antigens from WR available for priming between immunisations via the i.d. and i.p. routes are different which in turn influence the

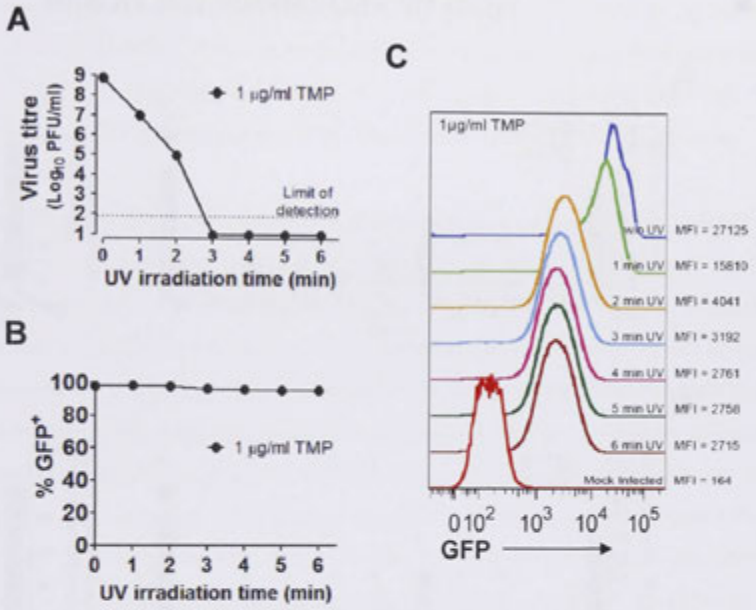


Figure 3-11| TMP treatment with brief UV irradiation inhibits VACV replication, but still allows early viral gene expression. 2×10^5 PFU/ml of WR-NP-S-GFP treated with 1 µg/ml of TMP in PBS (480 µl) was irradiated with UV light at 365 nm wavelength. At times indicated, 50 µl virus solution was taken for further experiments. (A) The replicative ability of the treated virus was examined by standard plaque assay. (B and C) The GFP expression of the treated virus in 293A cells at an m.o.i. of 5 after 6 h of infection was determined by flow cytometric analysis. (B) Percentages of cells expressing GFP after infection. (C) A histogram showing the level of GFP expression in the infected cells. This experiment was repeated twice; representative results from one experiment are shown.

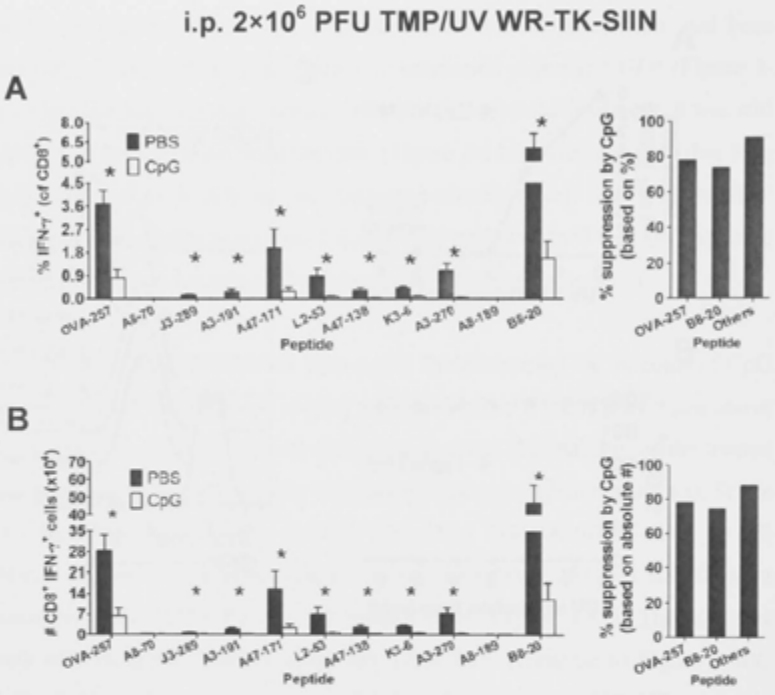


Figure 3-12| Viral replication ability dictates the inhibitory effect of CpG treatment on CD8$^+$ T cell responses after i.p. immunisation. Mice were treated with CpG or PBS and one day later they were immunised i.p. with 2×10^6 PFU WR-TK-SIIN that was treated with 1 μg/ml TMP and 3 min UV irradiation. The percentage (A) or absolute numbers (B) of CD8$^+$ T cell responses to the peptides indicated were measured in spleens 7 days later and are shown as means ± SEM on graphs on the left. Graphs on the right show the extent of suppression based on the data from the left. * denotes statistical significance ($p < 0.05$). Results are compiled from three independent experiments (n = 9).

inhibition effect of CpG. Previous studies have shown that WR replication and spread is relatively limited after i.d. injection into the ear pinnae compared with systemic routes (Tscharke and Smith, 1999; Fischer et al., 2011; Lin et al., 2013). Furthermore, CpG treatment can induce a robust innate immune responses to prevent the replication and dissemination of VACV in vivo (Rees et al., 2005; Belyakov et al., 2006). It was possible that these two factors together decrease the amount of viral antigens expressed in vivo and in turn compromise the $CD8^+$ T cell priming after immunisation of CpG-treated mice with WR via the i.d. route.

To test this hypothesis, the amount of infectious WR in various organs, including the known priming LNs, were examined after i.d. or i.p. immunisation of CpG-treated and control mice. Because priming occurs early after VACV immunisation (Norbury et al., 2002), one and three days post immunisation were chosen as the examination time points. After i.d. immunisation, there was no difference between the amount of infectious virus isolated from the infected ears of CpG-treated and control mice (Figure 3-13A). However, there was significantly lower amount of infectious virus (around 1.5 $\log_{10}$-fold reduction) in the draining cervical LNs. After i.p. immunisation with WR-TK-SIIN, although the averaged virus titres seemed to be lower in CpG-treated mice in the examined organs, the difference was only statistically significant in the spleen on day 3 (Figure 3-13B). These data imply that CpG treatment prevented the spread of WR and might compromise antigen loads in priming sites early after i.d. immunisation, but to a lesser extent for i.p. immunisation.

To investigate whether the reduction of the amount of WR alone might prevent an optimal priming of $CD8^+$ T cell responses after i.d. immunisation, a low dose of WR was used for immunisation. Mice were immunised with 2×10^3 PFU of WR, a 1000-fold reduction on the dose used in previous experiments. At the first day post immunisation, less than 2 PFU of virus was isolated from the draining cervical LNs (Figure 3-13C). This was much lower than that detected on day 1 in CpG-treated mice immunised with a high dose of virus (Figure 3-13A). The virus load in cervical LNs became more typical three days after immunisation with a low dose of WR. Despite the reduced virus levels in draining LNs on the first day, mice immunised with the low dose of WR had strong $CD8^+$ T cell responses to all

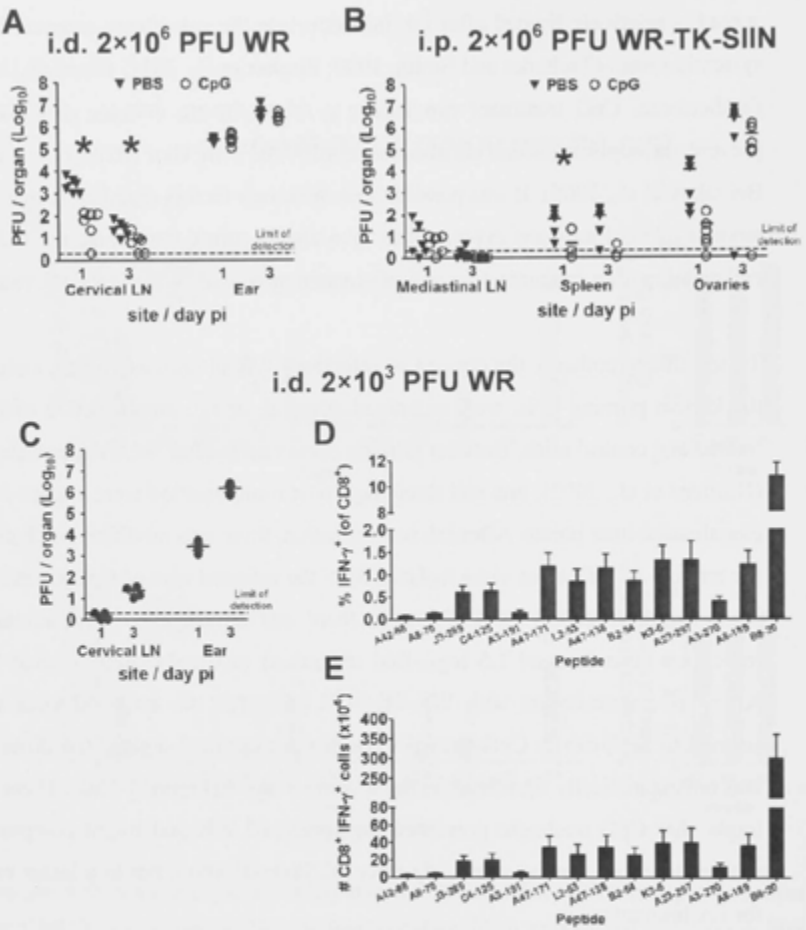


Figure 3-13| CpG treatment reduces VACV loads, but lower virus doses prime typical CD8$^+$ T cell responses. (A and B) CpG- or PBS-treated mice were immunised with 2×10^6 PFU VACV WR via the i.d route (A) or WR-TK-SIIN via i.p. route (B). Virus titers from organs as shown were determined by standard plaque assay 1 and 3 days post-immunisation. (C-E) Mice were immunised i.d. with 2×10^3 PFU WR. (C) Virus titres in LN and ear were measured 1 and 3 days after immunisation. Percentage (D) and absolute numbers (E) of splenic CD8$^+$ T cells producing IFN-γ after ex vivo stimulation with peptide indicated were measured 7 days after immunisation and are shown as mean ± SEM. (A and B) Data are combined from two independent experiments (n = 6). Data in (C-E) represent results from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

VACV epitopes, similar to those injected with 1000-fold more virus (Figure 3-13D and E, compared with control mice in Figure 3-8A and B). This experiment was not ideal because virus was available in the draining LNs at day 3 after low dose immunisation. This might contribute to $CD8^+$ T cell priming. Nevertheless, it has been suggested that the first 24 hours after immunisation is the most important time for priming $CD8^+$ T cells by VACV (Norbury et al., 2002). Also, by three days after immunisation, CpG significantly reduced the amount of virus in the spleens of mice immunised WR-TK-SIIN with via the i.p. route (Figure 3-13B). However, this did not affect the percentages of $CD8^+$ T cells that were specific to B8-20 and OVA-257 (Figure 3-10A). These arguments suggested that differences in virus levels at day 3 post immunisation might not be as relevant as those at earlier time points.

Collectively, CpG treatment reduced virus loads in the priming LNs and this might contribute to the reduced $CD8^+$ T cell priming after i.d. immunisation with WR. However, it seemed unlikely that this effect solely responsible for the strong inhibition of anti-WR $CD8^+$ T cell responses by CpG in this route.

3.2.8 Increased antigen dose partially overcomes the inhibition of $CD8^+$ T cell priming by CpG

The relationship between virus replication and CpG-induced inhibition of $CD8^+$ T cell responses explored in the previous sections suggests a role for antigen dose in influencing the outcome of CpG treatment. Therefore, it seemed likely that the inhibition of $CD8^+$ T cell responses by CpG treatment could be rescued simply by increasing the dose of antigen. This theory was tested by immunisation with a high dose of MVA. Here, CpG-treated and control mice were immunised twice with MVA-TK-SIIN, two days apart with 2×10^8 PFU via the i.p. route. This increased the MVA inoculum by 100-fold for each individual dose, as well as doubled the vaccination dose given as the dose used in Figure 3-10. From the result, the B8-20-specific response was still significantly suppressed by CpG treatment after this high-dose immunisation with MVA. However, the extent of the reduction decreased to around 45% (Figure 3-14A and B). In comparison, nearly 80% of B8-20-specific response was suppressed by CpG when the normal

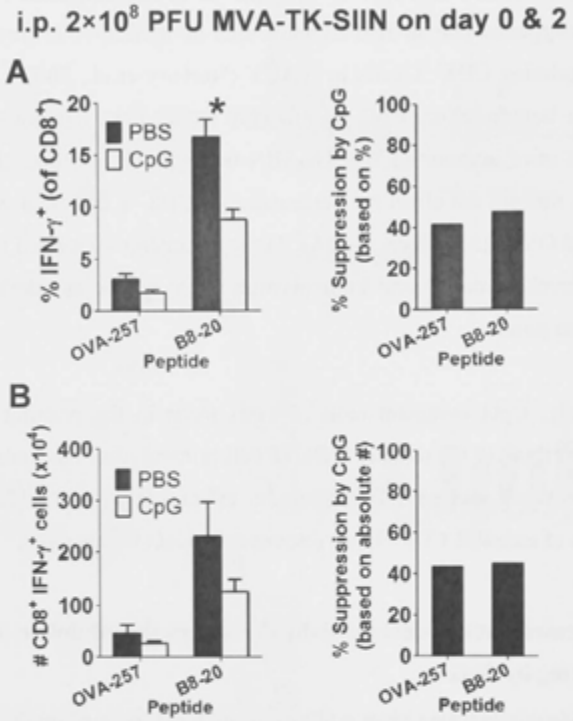


Figure 3-14| Immunisation with a higher dose of MVA overcomes the inhibitory effect of CpG treatment on CD8$^+$ T cell responses. Mice were immunised i.p. with 2×10^8 PFU of MVA-TK-SIIN on day 0 and day 2, starting from a day after CpG or PBS treatments. Percentages (A) and absolute numbers (B) of the CD8$^+$ T cells recognising OVA-257 and B8-20 were measured in spleens 7 days after immunisation. Results are shown as mean ± SEM on graphs on the left. Graphs on the right show the extent to which responses were suppressed in CpG compared with control treated mice, based on the data presented on the left. Results are compiled from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

dose of MVA was used (refer to Fig 3-10C and D). In addition, the OVA-257-specific response induced by the directly-presented OVA-257 minigene was no longer significantly lower in CpG-treated mice compared with control mice (Figure 3-14A and B). The apparent reduction of this response was around 40%, compared with 90% for the usual dose (refer to Fig 3-10C and D).

The ability of a high antigen dose to overcome the inhibition of cross priming by CpG treatment was then investigated. Here CpG-treated and control mice were immunised i.p. with 2.5×10^7 heat-inactivated WR-TK-OVA-infected cells, which was 25 times more than the dose used in the original experiments shown in Figure 3-7. A significantly lower response was observed for B8-20- but not OVA-257-specific $CD8^+$ T cells in CpG-treated mice compared with control mice (Figure 3-15). Furthermore, the suppression of $CD8^+$ T cell responses by the CpG treatment was reduced for both epitopes when mice were immunised with 2.5×10^7 infected cells (40-70%; right of Figure 3-15 A and B), as compared with experiments using a dose of 1×10^6 cells (more than 80%; Figure 3-7C and D). This finding with a high dose of antigen donor cells is similar to that using a dose of MVA inoculum (refer to Figure 3-14). Results in Figure 3-14 and 3-15 demonstrate that the extent to which CpG treatment inhibited $CD8^+$ T cell responses was sensitive to the antigen dose administered.

3.2.9 Cytc treatment fails to differentiate between direct and cross priming

The studies above indicate that CpG treatment only reflected antigen dose used, but not the $CD8^+$ T cell priming pathways. Therefore, a different strategy was needed for the original aim of this chapter, which was to dissect MHC-I presentation pathways for individual native VACV peptides. Cytc treatment was chosen as the alternative method because it has been published as an approach to selectively inhibit cross presentation (Lin et al., 2008).

The original report indicated that pre-treating mice once with 5 mg cytc one day before immunisation was enough to suppress cross priming in vivo (Lin et al., 2008). Here the length of cytc treatment was extended, in which mice were injected i.v. with 5 mg cytc for five consecutive days starting one day before

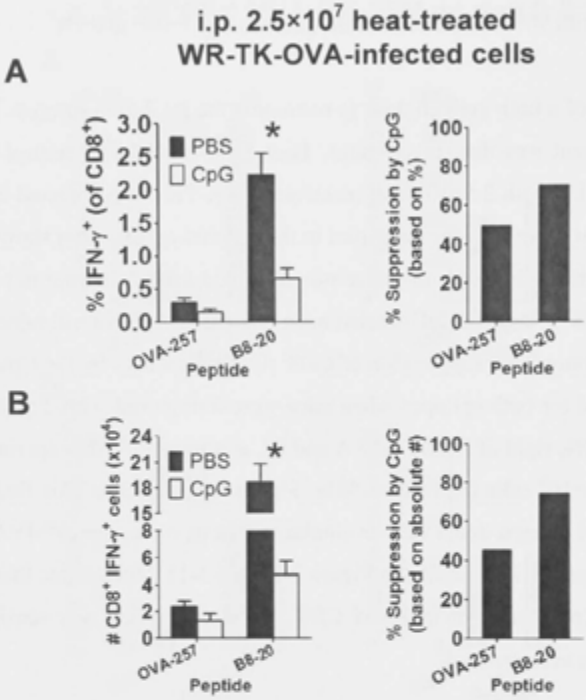


Figure 3-15| Increasing antigen dose partially overcomes the inhibitory effect of CpG treatment on cross priming. CpG- and PBS-treated mice were immunised i.p. with 2.5×10^7 heat-inactivated, WR-TK-OVA-infected 293A cells. Percentages (A) and absolute numbers (B) of the OVA-257- and B8-20-specific $CD8^+$ T cell responses were measured in spleens 7 days after immunisation and are shown as mean ± SEM on graphs on the left. Graphs on the right show the extent to which responses were suppressed in CpG compared with control treated mice, based on the data presented on the left. Results are combined from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

immunisation. This was to prolong its possible inhibitory effect on cross priming over the whole course of immunisation. Because cytc treatment has not been applied to the study of VACV before, its ability to stop cross presentation of VACV antigens in vivo was first examined. Figure 3-16 shows that cytc reduced $CD8^+$ T cell responses against multiple peptides elicited by VACV-infected cells. However, the inhibition seemed to be less than we observed for CpG treatment and most apparent reductions were not statistically significant.

Next, the effect of cytc on priming $CD8^+$ T cell responses during VACV immunisation was analysed. The VACV recombinants expressing the directly-presented OVA-257 minigene were used again here. This allowed us to determine if the treatment would affect direct priming. Results from Figure 3-17A show that $CD8^+$ T cell responses against OVA-257 and B8-20 induced by WR-TK-SIIN were enhanced by cytc treatment while other responses were not affected. This result contradicts the experiments with CpG as shown on Figure 3-10. For MVA-TK-SIIN, cytc caused a reduction in $CD8^+$ T cell responses against many peptides tested, including OVA-257 and B8-20 (Figure 3-17B). It was noticed that the A47-171-specific response was not affected by cytc here. To determine whether the outcomes of cytc treatment for WR and MVA were also dictated by their replicative ability, TMP/UV treatment (Section 3.2.6) was used again to stop WR-TK-SIIN from replicating in vivo. Similarly to the CpG experiment shown in Figure 3-12, priming of $CD8^+$ T cell responses by the non-replicating WR-TK-SIIN was suppressed by cytc for most peptides tested (Figure 3-17C), in a manner most similar to MVA, but not WR. It should be noted that in this experiment, the OVA-257-specific response was reduced by cytc, but the difference was not significant. Interestingly, the response to A47-171 was also inhibited here, different from MVA-TK-SIIN.

Overall, cytc clearly impairs direct priming of $CD8^+$ T cell responses during immunisation with non-replicating VACV. One possibility is that cytc may eliminate APCs that would normally be infected with VACV, which will compromise direct priming in vivo. In addition, the effects of CpG and cytc treatments on the $CD8^+$ T cell responses induced during WR immunisation were

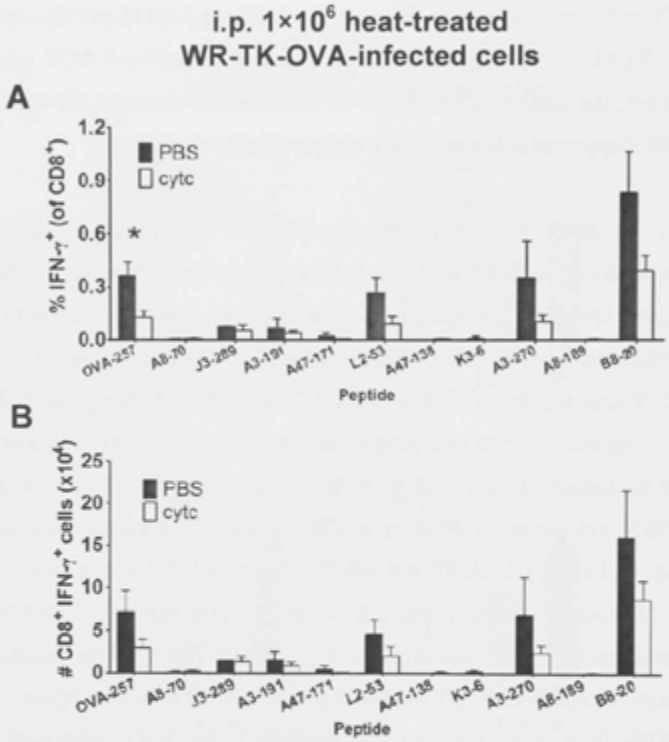


Figure 3-16| Cytc treatment reduces cross priming of CD8$^+$ T cell responses from VACV-infected cells. Cytc- or PBS-treated mice were immunised i.p. with 1×10^6 heat-inactivated WR-TK-OVA-infected 293A cells. Percentages (A) and absolute numbers (B) of CD8$^+$ T cell responses to peptides indicated were measured in spleens 7 days post immunisation by ex vivo peptide stimulation and ICS. Data are shown as means ± SEM and are combined from two independent experiments (n = 2 and 5 for PBS and cytc respectively). * denotes statistical significance ($p < 0.05$).

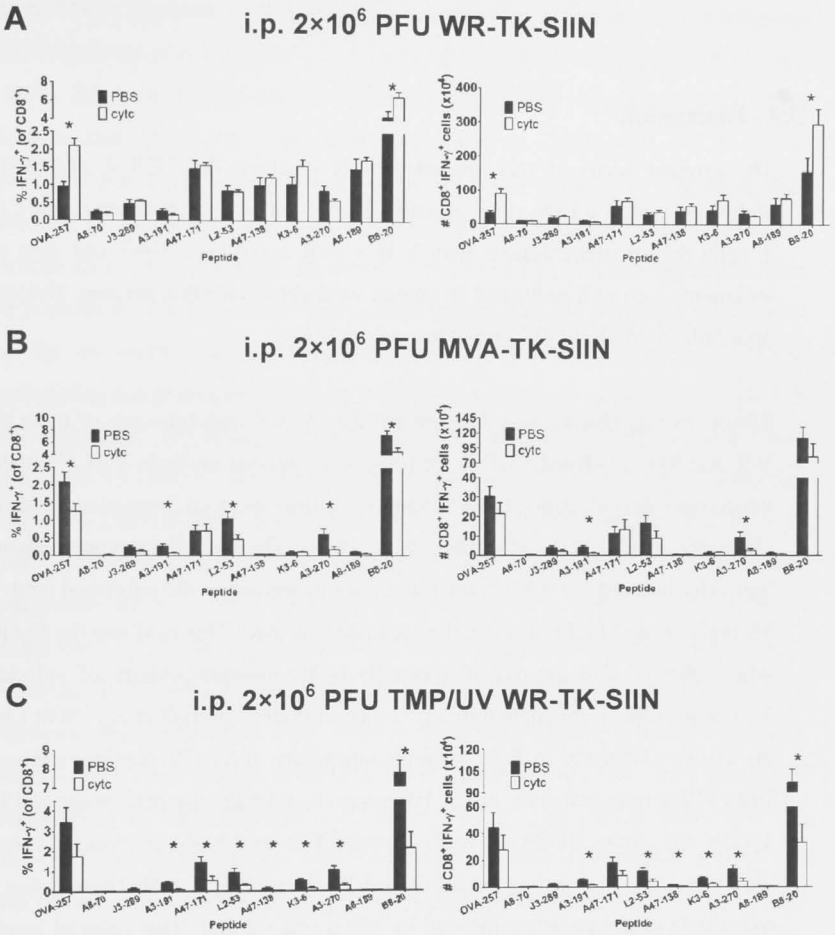


Figure 3-17| Cytc treatment does not dissect CD8$^+$ T cell priming pathway for VACV WR and MVA. Cytc-treated or control (PBS) mice were immunised i.p. with 2×10^6 PFU of WR-TK-SIIN (A), MVA-TK-SIIN (B) or WR-TK-SIIN treated with TMP/UV inactivation method as described in Figure 3-12 (C). Percentages (left) and absolute numbers (right) of CD8$^+$ T cell responses to the peptides indicated were measured in spleens 7 days later and are shown as means ± SEM. Results are combined from three (A) and two (B, C) independent experiments (A: n = 9; B and C: n = 6). * denotes statistical significance ($p < 0.05$).

not consistent. Therefore, cytc treatment does not provide insight into the pathways involved in priming anti-VACV $CD8^+$ T cells.

3.3 Discussion

The original focus of this chapter was to examine the MHC-I presentation pathways used by a large panel of native VACV antigenic peptides to prime $CD8^+$ T cells during immunisation with VACV WR and MVA. CpG and cytc pre-treatments, two well-published strategies, were chosen for this purpose. However, both failed to dissect $CD8^+$ T cell priming pathways.

Results in this chapter first demonstrate that dermal immunisation of mice with WR and MVA induced $CD8^+$ T cell responses against multiple VACV peptides which were first identified to be immunogenic from mice i.p. immunised with WR (Tscharke et al., 2005; Moutaftsi et al., 2006). The overall immunodominance hierarchy induced by WR via the i.d. route was similar to the published study by Moutaftsi et al. (2006) in which the i.p. route was used. This confirms the findings which showed that the overall hierarchy of the immunogenicity of individual VACV peptides is not altered by the choice of routes (Oseroff et al., 2008; Lin et al., 2013). Compared to WR, the immunogenicity of VACV peptides expressed from MVA were generally lower. For instance, although the percentage of $CD8^+$ T cells specific to B8-20 is similar between WR- and MVA-immunised mice, a closer examination of the total number of B8-20-specific T cells showed that the magnitude of the response induced by MVA was smaller. This reduced level of immunogenicity was more apparent for the subdominant antigens. A decreased overall anti-VACV $CD8^+$ T cell response induced during MVA immunisation compared to WR has been noted previously (Ramírez et al., 2000).

For two peptides, namely B2-54 and C4-125, the lack of responses can be explained by the absence of these two peptides from MVA. The B2 protein from MVA is truncated and it loses the carboxyl-terminal sequence, including the B2-54 peptide, when compared with WR (Antoine et al., 1998). It should be noted that a putative polypeptide, containing the B2-54 peptide, could be expressed

from the remanent of the MVA B2R gene (Antoine et al., 1998). However, the lack of B2-54-specific response implies that this protein might not be expressed from MVA. The entire C4L gene, which encodes the C4 antigen, is lost from the MVA genome as it is within one of the six large deletion regions (Antoine et al., 1998). Because no responses to B2-54 and C4-125 were induced by MVA, it implies that MVA does not encode cross-reactive mimitopes for these two peptides. For other antigens, one may argue that the level of expression and stability of the antigens may vary between WR and MVA due to small variations within the genes or promoter sequences (refer to Table A-2 in the Appendix). This is possible as MVA contains many small mutations within its genome, in addition to the six major deletions compared to WR. Thus, the efficiency of antigen processing and presentation may be affected. However, it is unlikely to explain all the differences. For instance, both the promoter sequence and the A8 amino acid sequence encoded by the VACV A8R gene from MVA and WR are identical (Table A-2 in Appendix) and so it is hard to explain the observed differences in its immunogenicity between WR and MVA with the antigen-intrinsic reason suggested above. Further, there are broad differences in the genetics between these two VACV antigens as a whole, which in turn dictates their virulence. Viral virulence is involved in modulating the magnitude of $CD8^+$ T cell responses. For instance, the B18R gene from VACV strain WR encodes a soluble and cell surface-associated IFN-I binding protein which is not functional in MVA (Symons et al., 1995; Alcamí et al., 2000; Waibler et al., 2009). When B18R is deleted from WR, it does not alter the replication ability of the virus in vitro, but the mutant is less pathogenic in vivo and induces a lower anti-VACV $CD8^+$ T cell response compared with the wildtype virus (Symons et al., 1995; Salek-Ardakani et al., 2011b). It has been shown that mice deficient in IFN-α/β receptor are more susceptible to infection with wildtype VACV strain WR (Muller et al., 1994). The $CD8^+$ T cell response in these mice against this VACV strain has not been reported, probably due to the high virulence of the virus in this mutant strain. Recently, a study shows that MVA can induce a functional $CD8^+$ T cell response in IFN-α/β receptor-deficient mice (Volz et al., 2014). However, a direct comparison between wildtype and the transgenic mice has yet to be reported. It should be noted that, as discussed in section 1.5.3, direct IFN-I signalling is essential for the expansion of $CD8^+$ T cells after priming (Thompson et al., 2006).

In addition, WR induced a stronger anti-viral $CD8^+$ T cell response than VACV strains that are less virulent (Ramírez et al., 2000; Salek-Ardakani et al., 2011a)., It has been shown that the less virulent strains are less able to fully activate multiple co-stimulatory signalling pathways (Salek-Ardakani et al., 2011b). These antigen-extrinsic factors which are defined by the interaction between the virus and host are clearly important in dictating how well a $CD8^+$ T cell response is primed during VACV immunisation.

We have developed an in vivo cross priming assay here based on immunisation with VACV-infected cells. We found that heat inactivation is a suitable method for inhibiting residual virus on infected cells for use in a cross priming assay. Psoralen/UV treatment, which crosslinks the DNA genome of VACV after UV irradiation, inhibited viral replication in our studies, but failed to completely inactivate OVA-257 minigene gene expression from WR-TK-SIIN. It has been shown that antigen presentation from a VACV-expressed minigene is not as severely affected by this treatment as a full-length antigen (Fischer et al., 2007). This is most likely because of a lower probability of crosslink formation within shorter, compared to longer, genes. Our results confirmed that it is difficult to eliminate the minigene expression and presentation from WR-TK-SIIN with psoralen/UV treatment. We were concerned that this treatment might not be reliable and therefore it was not chosen for use in our cross presentation assay. Heat inactivation, on the other hand, causes denaturation of VACV antigens. It has been shown that when VACV was heated to 60°C, there was a rapid inactivation of most virus particles within 10 min of treatment, followed by a gradual decline of infectivity of residual virus particles that seemed to be less thermosensitive (Kaplan, 1958). More importantly, after 60°C heat treatment, infectivity of VACV was reduced by more than 10^7-fold as shown in multiple studies (Kaplan, 1958; Woodroofe, 1960; Madeley, 1968). In addition, this treatment abrogates the ability of VACV to transcribe and translate viral genes within cells (Harper et al., 1978). Our studies with WR-TK-SIIN, which show that heating VACV to 60°C for 60 min sufficiently abolishes both virus replication and MHC-I presentation of the OVA-257 minigene, confirm these early reports. Indeed, heat-treated cells that had been infected with WR-TK-OVA, but not WR-TK-SIIN, primed an OVA-257-specific response in vivo, in accordance with a

previous finding which utilised germicidal UV irradiation to inactivate VACV-infected cells (Norbury et al., 2004). In addition, the specificity of $CD8^+$ T cell responses induced by heat-inactivated WR-infected cells was similar to the responses elicited with germicidal UV-inactivated cells infected with WR-TK-OVA (Lev et al., 2009). It should be noted that heating antigens could result in antigen aggregation (Speidel et al., 1997; Cho et al., 2003). This could influence how an antigen is cross presented. To test this possibility, a 293A cell line stably expressing VACV B8 protein could be treated with heat inactivation. These treated cells could then be used to immunise mice. Any differences in the B8-20-specific $CD8^+$ T cell response induced by the heat-treated cells compared to the untreated cells will indicate that the inactivation treatment affects how an antigen is processed for cross priming. Transgenic mice lacking components essential for certain cross presentation pathways (such as cathepsin S-deficient mice) could also be used to determine the processing pathway involved. Overall, data presented in this chapter support the validity of the use of heat treatment to eliminate residual virus on the infected cells used in our cross priming experiments.

When we examined which antigens are cross presented from VACV-infected cells, we found that peptides originating from the early (J2-289, L2-53, K3-6, A47-171 and B8-20), intermediate (A3-191 and A3-270) and late (A42-88) classes of VACV gene products are all immunogenic in this context. Interestingly, although both $CD8^+$ T cell epitopes from A47 (A47-138 and A47-171) are immunogenic during WR immunisation, only A47-171, but not A47-138, can be cross presented from infected cells to prime $CD8^+$ T cells. In contrast, $CD8^+$ T cells that recognised the A3-191 and A3-270 peptides originating from the A3 antigen can be primed via cross presentation from VACV-infected cells. This implies that cross presentation of a peptide is not only influenced by the nature of the cross-presented antigen, but also affected by either the characteristic of the presented peptide or the processing of the peptide from the surrounding amino acid sequences of the antigen. It is also noticed that responses against subdominant peptides induced by cross priming from MVA-infected cells were lower than those of WR-infected cells after i.p. immunisation. Although many mammalian cell lines, including the human 293A cell line used as antigen donor

cells here, are not permissive for MVA replication, late genes from MVA are still expressed efficiently within these cells (Sutter and Moss, 1992; Carroll and Moss, 1997; Ludwig et al., 2005). In addition, it has been shown that MVA expressed a similar level of β-galactosidase antigen when driven by a VACV late promoter in infected 293A cells, compared to WR (Sutter and Moss, 1992). This suggested that a similar level of antigens can be expressed by WR and MVA in the antigen donor cells used here. However, we could not rule out that native VACV antigens, which are the focus of this study, might be expressed differentially by these two VACV strains.

This cross priming assay successfully demonstrated that some, but not all, antigens can be cross presented from VACV-infected cells. However, there are several limitations related to this cross priming assay. Firstly, our cross priming assay is different from the naturally-occurring cross priming during virus immunisation. This is because the infected antigen donor cells were heat-treated and so no further antigen expression was allowed in our assay. Secondly, there may be unmapped VACV $CD8^+$ T cell epitopes that are exclusively cross presented. $CD8^+$ T cell responses against these peptides might have been missed. Thirdly, VACV antigens may be available in various forms for cross presentation, not only associated with infected cells. For instance, secreted VACV antigens may be cross presented as soluble antigens. VACV B8 antigen, a secreted IFN-γ receptor (Alcami and Smith, 1995; Sroller et al., 2001; Verardi et al., 2001; Symons et al., 2002), may be able to be cross-presented in this form. Interestingly, several of the antigens that are cross presented from VACV-infected cells are found within virion particles, including A3, A42, and J3 antigens (Jensen et al., 1996; Chung et al., 2006; Yoder et al., 2006; Resch et al., 2007). The hypothesis that these antigens can be cross presented from inactivated virions to prime $CD8^+$ T cell responses are examined in Chapter 6.

After finding that some VACV antigens could be cross presented from infected cells, the next question was how native VACV antigens are presented during VACV immunisation. Here the CpG and cytc treatments were used. However, both methods failed to determine the priming mechanisms for anti-VACV $CD8^+$ T cells in mice. Firstly, studies here show that CpG treatment inhibits direct priming

by VACV when the dose of antigen is limited, especially during immunisation via the local dermal route or with non-replicating VACV. Secondly, the extent of CpG inhibition of cross priming from virus-infected cells is not absolute. Rather it depends on the quantity of antigen donor cells used. These findings highlight the difficulty in examining direct and cross priming during a viral infection with this method. When infectious virus is used, replication occurs and the antigen dose produced can be very high. This effect would be even more prominent if the infection can spread to other organs, which is exactly what occurred during i.p. immunisation with VACV WR (Buller et al., 1985; Lin et al., 2013). Disruption of the VACV TK gene by insertion of foreign antigen-expressing constructs into the genome of WR decreases the virulence of the resulting recombinants (Buller et al., 1985). However, these recombinants, such as WR-TK-SIIN used in this study, can still spread to multiple internal organs after i.p. injection (Buller et al., 1985; Ramírez et al., 2000; Benning and Hassett, 2004; Gómez et al., 2007). In contrast, non-replicating viruses, such as MVA, provide relatively lower amounts of antigen in vivo (Ramírez et al., 2000; Gómez et al., 2007). Similarly, the in vivo cross priming assay used in this thesis can also be considered to provide a low antigen dose for priming. This is because a finite number of antigen donor cells were used and no antigens were expressed further within the cells after heat treatment. Methods such as the CpG treatment, which is sensitive to antigen levels as shown in Sections 3.2.6 and 3.2.8, might suppress $CD8^+$ T cell responses induced by low antigen doses to a greater extent than those associated with high antigen doses. This might explain the results of experiments using CpG which at first glance imply priming by MVA by any route and WR by i.d. injection in the ear requires cross presentation.

Our data do not reveal how CpG treatment influences anti-VACV $CD8^+$ T cell priming. Extensive systemic CpG injection results in a significant change in the host's immune system. This includes splenomegaly and a change of the composition of lymphocytes in the spleen (Sparwasser et al., 1999; Heikenwalder et al., 2004). Importantly, the organisation of secondary lymphoid organs is disrupted in mice treated with CpG (Heikenwalder et al., 2004). In addition, CpG treatment affect the migration of DCs in vivo (Behboudi et al., 2000; Yang et al., 2005; Constabel et al., 2009). The latter two effects might negatively influence

how well DCs can be infected by virus in vivo. They might also prevent cell-to-cell contacts between $CD8^+$ T cells and antigen-presenting DCs in vivo, which are required for priming. Furthermore, it has been shown that CpG treatment increases expression of indoleamine 2,3-dioxygenase and activation of regulatory T cells, which results in a suppression of $CD8^+$ T cell immunity (Wingender et al., 2006; Baban et al., 2011). Finally, because CpG treatment reduces VACV loads, it might decrease the antigen dose available for priming, especially at sites where VACV spread is restricted, such as i.d. immunisation.

Results in Figure 3-8 and 3-10 show that CpG caused a differential suppression of $CD8^+$ T cell responses across different VACV peptides. The mechanism behind this was not further pursued. Interestingly, $CD8^+$ T cell epitope-specific effects have been observed during co-administration of TLR ligands with LCMV (Siddiqui and Basta, 2011), suggesting this phenomenon is unlikely to be specific to VACV. It is possible that DCs matured by CpG in vivo might have a different functionality and this might result in an alteration of their ability to express, process and present individual antigens via direct presentation pathway. The high level of MHC-I presentation of the immunodominant B8-20 peptide and the OVA-257 minigene (Princiotta et al., 2003; Croft et al., 2013) might also partially compensate for the general reduction of $CD8^+$ T cell priming caused by CpG treatment, allowing $CD8^+$ T cell responses to these two peptides to be induced. Further, the relatively high frequency of B8-20-specific $CD8^+$ T cells in the naive repertoire might play a role as well (Flesch et al., 2010). However, it should be noted that CpG reduced the overall anti-VACV $CD8^+$ T cell immunity when the total number of responding $CD8^+$ T cells was measured, demonstrating its inhibition effect on $CD8^+$ T cell priming in general. Indeed, Wilson et al. (2006) demonstrated that CpG inhibits the $CD8^+$ T cell response after HSV infection in mice although this outcome was attributed solely to the ability of CpG to inhibit cross presentation by the authors.

Wilson et al. (2006) showed that direct priming of $CD8^+$ T cells against OVA expressed endogenously in DCs was not affected by CpG in CD11c-OVA transgenic mice, where the OVA expression (as a membrane-bound antigen) is under the control of the CD11c promoter. In this model, OVA-257 should be

continuously presented by MHC-I on the DC surface. Maturation of DCs by CpG might simply enhance the stability of this established presentation (Chow et al., 2002; Ackerman and Cresswell, 2003). In addition, CpG might further enhance expression of co-stimulatory molecules on these presenting DCs (Hemmi et al., 2000; Sparwasser et al., 2000), resulting in better $CD8^+$ T cell activation. This is different from the situation of the immunisation of mice after CpG treatment, where a virus must infect and have its antigens expressed, processed and presented by mature DCs. Moreover, the CD11c-OVA transgenic mice used by Wilson et al. (2006) are not well characterised. In addition to DCs, other $CD11c^+$ cells would also be expected to express the OVA antigen in these mice. This could complicate the interpretation of the results. Furthermore, there are in vitro studies suggesting that presentation pathways cannot be separated simply based on DC maturation status. For instance, mature DCs generated from in vitro culturing with CpG could still cross present soluble or cell-associated antigens (Datta et al., 2003; de Brito et al., 2011). In addition, it has been shown that in vivo CpG pre-treatment does not affect cross priming of $CD8^+$ T cell responses against soluble antigens (Drutman and Trombetta, 2010). This would further complicate the interpretation of data even if direct presentation was not affected. This demonstrates the complexity of models for dissecting antigen presentation that are based on the DC maturation states.

Experiments with a different strategy, the cytc treatment, demonstrate that this treatment was also unable to show any insight into the priming mechanisms of anti-VACV $CD8^+$ T cell responses. In vivo cytc treatment causes a depletion of a subset of $CD8\alpha^+$ DCs that are responsible for phagocytosing exogenous antigens for cross presentation (Lin et al., 2008; Qiu et al., 2009). The $CD8\alpha^+$ DC subset has also been implicated in $CD8^+$ T cell priming by WR (Belz et al., 2004; He et al., 2006). Together with the intravital observation of direct priming of $CD8^+$ T cells by WR-infected DC (Norbury et al., 2002; Hickman et al., 2008), it is reasonable to assume that the $CD8\alpha^+$ DC subset is required for direct priming of anti-WR $CD8^+$ T cells. Therefore, it was unexpected when it was found that $CD8^+$ T cell responses to B8-20 and the directly-presented OVA-257 minigene induced by WR-TK-SIIN were enhanced after cytc treatment. It could be that the induction of DC apoptosis by cytc might disturb the organisation of secondary

lymphoid organs, similar to the CpG treatment. It is also possible that unknown signals might be generated after the elimination of DCs, which might modulate $CD8^+$ T cell responses. However, it should be noted that the effect is peptide-specific as responses against other tested VACV peptides were not affected. More importantly, cytc treatment, similar to CpG, impairs direct presentation. This is demonstrated by its inhibition on the direct priming of $CD8^+$ T cells specific to the OVA-257 minigene expressed from MVA-TK-SIIN and the non-replicating TMP/UV-inactivated WR-TK-SIIN. A recent report by Kastenmüller et al. (2013) used in vivo imaging and found evidence for direct $CD8^+$ T cell priming by MVA-infected DCs in draining LNs. If priming of $CD8^+$ T cells specific to antigens expressed from MVA and TMP/UV-inactivated WR requires $CD8\alpha^+$ DCs (as for WR), an elimination of these cells by cytc might result in poor direct priming. Although we showed that cytc suppressed cross presentation of VACV-infected cells in vivo, we did not directly demonstrate whether cytc could deplete cross-priming $CD8\alpha^+$ DCs. It is possible that the effects observed could be a side effect. This could be easily tested by measuring the numbers of $CD8\alpha^+$ DCs in mice with and without cytc treatment and by determining the cross priming ability of the remaining $CD8\alpha^+$ DCs in cytc-treated mice ex vivo. Overall, results in this chapter show that the CpG and cytc treatments altered the $CD8^+$ T cell priming in a way that was independent of the presentation pathways. This is probably because these treatments do not simply act on the presentation pathway directly, but via modifications of the overall immune system.

The results presented here have several implications on antigen presentation in general. Firstly, our findings may not simply be limited to treatments with CpG and cytc, but may also be applicable to other TLR ligands that have been suggested to inhibit cross presentation in vivo, such as lipopolysaccharide (a TLR-4 ligand) and synthetic dsRNA polyinosinic: polycytidylic acid (poly(I:C); a TLR-3 ligand; Wilson et al., 2006; Bouvier et al., 2011). Recently, it has been shown that pre-treating DCs with pure LPS actually enhances in vitro cross presentation and peptidoglycan, a contaminant found during normal LPS preparation, is the chemical responsible for inhibiting cross presentation after DC maturation (Wagner and Cresswell, 2012). Secondly, we also believe that the findings shown in this chapter are not specific for VACV, but can apply to other viruses. As such,

experiments in which cross presentation is targeted by these methods should be analysed with caution.

Interestingly, data in Figures 3-9 and 3-10 show that strong OVA-257-specific responses were induced in control mice after i.d. and i.p. immunisation with WR-TK-SIIN and MVA-TK-SIIN. These results demonstrate that antigens expressed from these two VACV strains can be directly presented effectively for $CD8^+$ T cell priming in vivo. This confirms reports for WR (Lawson et al., 1994; Restifo et al., 1995; Deng et al., 1997; Tobery and Siliciano, 1997; Norbury et al., 2004; Tewalt et al., 2009), but contradicts that of Gasteiger et al. (2007), which suggested that $CD8^+$ T cell responses against antigens expressed from MVA are induced by cross priming. Further comparisons of minigenes versus full-length antigens expressed from WR and MVA are examined in Chapter 4.

In conclusion, the presentation mechanism for individual native VACV peptides expressed from VACV strains WR and MVA were not successfully determined with CpG and cytc treatments in this chapter. This is mainly due to the complex effects of these treatments in vivo independent of antigen presentation pathways.

Chapter 4 Effective $CD8^+$ T cell priming against minigenes expressed from modified vaccinia virus Ankara depends on the insertion site in the viral genome

4.1 Introduction

In the previous chapter, we attempted to dissect how native VACV peptides are presented using CpG and cytc treatments. Although these two strategies failed to provide any insights into the presentation pathways involved in priming anti-VACV $CD8^+$ T cells, it was noted that OVA-257-specific $CD8^+$ T cell responses were induced by the directly-primed OVA-257 minigene expressed from VACV WR and MVA. The effective direct priming of the OVA-257 minigene expressed from MVA is surprising because it contradicts the study of Gasteiger et al. (2007).

As discussed in Section 1.7.4, it is generally accepted that rapidly-degraded antigens and cytosolic or ER targeted minigene constructs facilitate direct presentation. Increasing the rate of proteasomal degradation of an antigen enhances the efficiency of direct presentation (Townsend et al., 1988; Rodriguez et al., 1997; Princiotta et al., 2003). Another strategy to enhance direct presentation is to bypass the requirement for protolytic processing entirely. As discussed in Section 1.7.4, peptides expressed as cytosolic minigenes require minimal antigen processing (to remove the methionine at the amino-terminus encoded by the start codon) while ER-targeted minigenes can even bypass the need for TAP translocation to bind to MHC-I (Eisenlohr et al., 1992b; Bacik et al., 1994; Antón et al., 1997; Porgador et al., 1997; Fu et al., 1998; Princiotta et al., 2003; Lev et al., 2008).

Most of the above methods to enhance the efficiency of direct presentation originated from studies with VACV, mainly with strain WR. Foreign antigens expressed from WR in forms designed to facilitate direct presentation allow effective priming of $CD8^+$ T cells in vivo. In many cases they are found to be more immunogenic than the full-length stable antigens (Irvine et al., 1995; Restifo et al., 1995; Tobery and Siliciano, 1997; Fu et al., 1998; Tobery and Siliciano, 1999; Norbury et al., 2004). In these studies, the in vitro direct presentation rate and the in vivo $CD8^+$ T cell priming ability are clearly correlated. However, the study by Gasteiger et al. (2007) demonstrated that there was an opposite correlation for antigens expressed from MVA. The cytosolic OVA-257 minigene expressed from MVA induced a significantly lower OVA-257-specific $CD8^+$ T

cell response compared to the full-length antigen, in spite of its more efficient direct presentation in vitro (Gasteiger et al., 2007). The investigators also noticed a similar result when they looked at the model tumor antigen Tyr, which contained a human MHC-I HLA-A*0201-restricted Tyr-369 epitope (Wölfel et al., 1994; Mosse et al., 1998). Gasteiger et al. (2007) found that enhancing the degradation of Tyr by fusing it to ubiquitin increased the in vitro direct presentation efficiency. However, this modification reduced the ability of the antigen to prime Tyr-369-specific $CD8^+$ T cell response in transgenic HHD mice expressing HLA-A*0201.

These findings by Gasteiger et al. (2007) suggest that constructs that facilitate direct priming do not allow effective priming in vivo when expressed from MVA. However, there are several caveats in their studies. Firstly there was no direct comparison with the relevant WR recombinants. WR recombinants expressing Tyr either as a minigene or as a full-length antigen have been generated and both allow induction of a Tyr-369-specific $CD8^+$ T cell response in mice (Bullock et al., 2000; Palmowski et al., 2002). However, different techniques were used to measure the $CD8^+$ T cell responses and so the relative immunogenicity between the Tyr-369 minigene and the full-length Tyr antigen could not be compared. Secondly, Gasteiger et al. (2007) focused on MVA recombinants that express foreign antigens from an insertion site that is not available in the WR genome. For most WR recombinants, including those used to examine the immunogenicity of minigenes and rapidly-degraded constructs as discussed above, the transgenes are commonly inserted into the J2R gene, which encodes the VACV TK protein. This gene locus is chosen as the insertion site because there is a well-established method to allow the selection of final recombinants that are TK^- (Chakrabarti et al., 1985). This involves the use of 5-bromodeoxyuridine (BUdR) to suppress the replication of TK^+ wildtype virus during the generation of recombinant virus with a TK^- phenotype (Mackett et al., 1982; Mackett et al., 1984; Chakrabarti et al., 1985). On the other hand, Gasteiger et al. (2007) generated their MVA recombinants by inserting foreign antigens into the delIII region, one of the six large deletions in the MVA genome compared to those of other VACV strains (Section 1.9.2). The delIII region of MVA corresponds to a genomic segment encoding four functional proteins in WR and as such cannot be used as an

insertion site for WR. A previous report found that the expression level of a given transgene from WR depends on the insertion site used (Coupar et al., 2000). Thus, results from Gasteiger et al. (2007) should not be directly compared with studies that used WR recombinants. Thirdly, only two model antigens were examined for MVA in one single study. More examples are needed to test whether the findings of Gasteiger et al. (2007) can be generalised to other antigens.

In this chapter, the immunogenicity of a number of recombinant antigens expressed as minigenes from VACV WR and MVA were compared with the full-length antigens. For each set of recombinants used, the inserted antigen was expressed by the same promoter from the same insertion site. This allowed a direct comparison between the two VACV strains. We found that minigenes of these antigens expressed from WR and MVA allowed effective in vitro direct presentation. Importantly, minigenes expressed from both VACV strains were as immunogenic as, if not better than, full-length antigens in inducing $CD8^+$ T cell immunity in vivo. Further investigations were conducted to understand the disparity between our results and those published. Studies with the OVA-expressing MVA recombinants generated by Gasteiger et al. (2007) and the related OVA-expressing MVA recombinants demonstrated that the insertion site and the function of VACV TK play a role in dictating the immunogenicity of the foreign antigen.

4.2 Results

4.2.1 The immunogenicity of the immunodominant HSV gB-498 epitope when expressed as a minigene from VACV WR and MVA

(Viruses used in this chapter are listed in Table A-3 in the Appendix)

The immunodominant $CD8^+$ T cell epitope from HSV-1 was chosen for the first set of experiments. The peptide is known as gB-498 and it is processed from the HSV gB antigen, with the amino acid sequence SSIEFARL (Hanke et al., 1991; Bonneau et al., 1993). This peptide is presented by MHC-I H-$2K^b$. The gB antigen is localised to the HSV-1 virion envelope and is important for virus entry into cells (Sarmiento et al., 1979; Sarmiento and Spear, 1979; Cai et al., 1988). During

an acute HSV-1 infection in C57Bl/6 mice, around 5-10% of total splenic $CD8^+$ T cells recognise gB-498, representing at least 45% of the total anti-HSV-1 $CD8^+$ T cell response (Wallace et al., 1999; St. Leger et al., 2011). HSV-2 also expresses this peptide and it is recognised by the $CD8^+$ T cells induced during HSV-2 infection (Bonneau et al., 1993; Tang and Rosenthal, 2010).

WR recombinants expressing the full-length HSV gB protein or an ER-targeted gB-498 minigene, namely WR-Full-gB and WR-ESmini-gB respectively, were available (Blaney et al., 1998; Lin et al., 2013). The gB constructs of both recombinants are expressed under the control of the early/late VACV p7.5 promoter (Cochran et al., 1985) from the VACV TK locus. The immunogenicity of the constructs expressed from these WR recombinants has been studied in mice. Compared with the full-length gB, the ER-targeted minigene induces a significantly higher gB-498-specific $CD8^+$ T cell response when expressed from WR (Lin et al., 2013). In addition, the response induced by the minigene is at a level similar to that of the immunodominant VACV B8-20 epitope (Lin et al., 2013). We were interested in the immunogenicity of this highly immunogenic HSV antigen when expressed from MVA. In order to allow a direct comparison of the results between WR and MVA, two new MVA recombinants were designed and generated. They express either a full-length gB protein or an ER-targeted gB-498 minigene by the same p7.5 promoter and from the same insertion site (VACV TK locus) as the set of WR recombinants mentioned above. The MVA recombinants are called MVA-Full-gB and MVA-ESmini-gB respectively.

4.2.1.1 Direct presentation of gB-498 is enhanced when it is expressed as an ER-targeted minigene from VACV WR and MVA

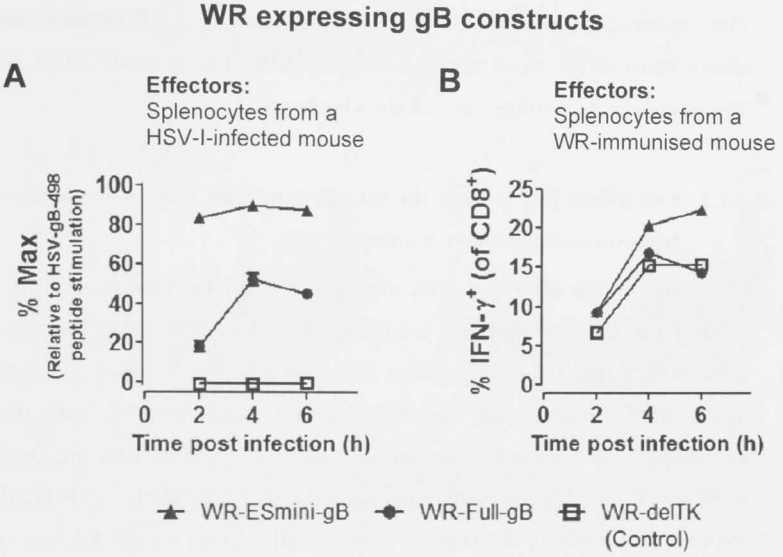
It is generally accepted that minigenes allow better direct presentation in vitro than full-length antigens when expressed from VACV, but it has not been empirically demonstrated for the HSV gB antigen. Therefore, this question was first examined using the gB-expressing WR recombinants and the newly-made MVA recombinants. Because there is no available reagent for direct measurement of gB-498/H-$2K^b$ complexes, we set up an in vitro direct presentation assay using re-stimulation of splenocytes from a mouse infected with HSV-1 strain KOS as a readout. In brief, DC2.4 cells, a DC-like cell line generated from C57Bl/6 mice

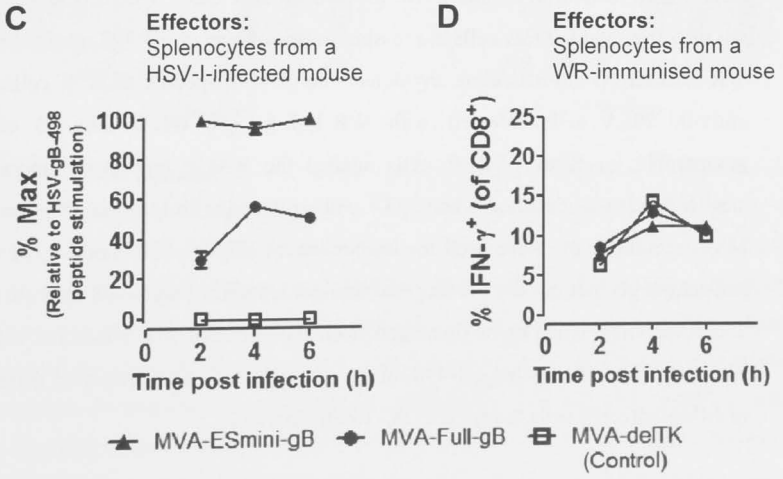
(Shen et al., 1997), were used as stimulators. These cells were infected with the gB-expressing VACV recombinants. The infected cells were then used to induce IFN-γ production in $CD8^+$ T cells from a HSV-immunised mouse during co-culturing in the presence of BFA. Standard ICS was then performed to measure the percentage of $CD8^+$ T cells expressing IFN-γ after stimulation.

The percentage of HSV-immune $CD8^+$ T cells activated reflects the direct presentation level of the gB-498 peptide on the infected DC2.4 cells. Although this assay has the possibility of detecting the presentation of other antigenic epitopes available in the full-length gB protein, the MHC-I binding affinities of the other known epitopes from gB are much lower than the immunodominant gB-498 epitope (St. Leger et al., 2011). More importantly, these minor epitopes only constitute a very small proportion of the anti-HSV $CD8^+$ T cell response induced in HSV-immunised mice (St. Leger et al., 2011). Also, these $CD8^+$ T cells would favour recognition of the full-length gB only, but not the gB-498 minigene. Therefore, they should not affect the analysis of this assay.

Results of this in vitro direct presentation assay are shown in Figure 4-1. Firstly, DC2.4 cells infected with WR-delTK and MVA-delTK, two control recombinants which do not express any gB constructs, did not stimulate the HSV-specific $CD8^+$ T cells (Figure 4-1A and C). This demonstrates that there is no cross-reactivity of HSV-specific $CD8^+$ T cells to VACV antigens. Secondly, DC2.4 cells infected with VACV expressing the gB-498 constructs were able to re-stimulate the HSV-immune $CD8^+$ T cells in vitro, in spite of the differences in the re-stimulation levels between different constructs. Kinetically, the re-stimulation of HSV-immune $CD8^+$ T cells by DC2.4 cells infected with WR-ESmini-gB and MVA-ESmini-gB reached the maximal level after two hours of infection and remained at this high level for the following four hours (Figure 4-1A and C). In comparison, the stimulation level by DC2.4 cells infected with WR-Full-gB and MVA-Full-gB increased gradually in the first four hours and then levelled off. However, even at the maximal level, DC2.4 cells infected with VACV expressing the full-length gB protein only stimulated around 50-60% of the HSV-immune $CD8^+$ T cells compared to those infected with VACV expressing the gB-498 minigene. As a

Figure 4-1| HSV gB-498 expressed as an ER-targeted minigene is directly presented more effectively than as a full-length antigen when expressed from WR and MVA. DC2.4 cells (simulators) were infected with the indicated recombinant WR (A and B) or MVA (C and D) for 5 h. They were then co-cultured with splenocytes (effectors) from mice immunised with either HSV-1 strain KOS (A and C) or VACV WR (B and D) seven days earlier. A stimulator:effector ratio of 1:5 was used. BFA was added 1 h after cells were mixed, followed by another 3 h of co-culture. ICS was then performed to measure the percentage of $CD8^+$ T cells that produced IFN-γ. Standard ICS assay with a synthetic gB-498 peptide was also performed in parallel to measure the maximum possible gB-498-specific T cell response. (A and C) Data show the level of gB-498-specific $CD8^+$ T cell activation by the infected DC2.4 cells, standardised to the percentage of maximal activation induced by the synthetic gB-498 peptide. (B and D) Data show the percentages of splenic $CD8^+$ T cells from a WR-immunised mouse activated by the indicated infected DC2.4 cells. Data for the gB-expressing recombinants represent mean ± SEM from triplicate samples while data for the control viruses represent single measurements. Data are representative of two independent experiments.


WR expressing gB constructs
A
Effectors:
Splenocytes from a
HSV-I-infected mouse
% Max
(Relative to HSV-gB-498
peptide stimulation)
100
80
60
40
20
0
0 2 4 6
Time post infection (h)
B
Effectors:
Splenocytes from a
WR-immunised mouse
% IFN-γ+ (of CD8+)
25
20
15
10
5
0
0 2 4 6
Time post infection (h)
WR-ESmini-gB
WR-Full-gB
WR-delTK
(Control)


MVA expressing gB constructs
C
Effectors:
Splenocytes from a
HSV-I-infected mouse
% Max
(Relative to HSV-gB-498
peptide stimulation)
100
80
60
40
20
0
0 2 4 6
Time post infection (h)
D
Effectors:
Splenocytes from a
WR-immunised mouse
% IFN-γ+ (of CD8+)
25
20
15
10
5
0
0 2 4 6
Time post infection (h)
MVA-ESmini-gB
MVA-Full-gB
MVA-delTK
(Control)

control, these infected DC2.4 cells stimulated VACV-specific $CD8^+$ T cells adequately, suggesting that the cells are infected comparably (Figure 4-1B and D). This experiment demonstrates that gB-498 expressed as an ER-targeted minigene allows better direct presentation on infected cells than as a full-length antigen. This phenomenon is irrespective of virus background.

4.2.1.2 Full-length gB, but not the gB-498 minigene, can be cross presented from recombinant VACV-infected cells.

The ability of the gB-498 peptide expressed as various forms from VACV for $CD8^+$ T cell cross priming was examined. Here the in vivo cross priming assay using VACV-infected antigen donor cells established in Section 3.2.3 was used again. MHC-I-mismatched human 293A cells were infected with the gB-expressing VACV recombinants for six hours, followed by heat inactivation at 60°C for 60 min. Mice were then immunised with these infected cells via the i.d. route in the ear pinnae. Responding $CD8^+$ T cells against the gB-498 and various VACV epitopes were measured seven days later.

The data in Figure 4-2A shows that 293A cells infected with WR-Full-gB induced a response of approximately 0.2% of $CD8^+$ T cells that were specific to gB-498. This was equivalent to around 1×10^4 gB-498-specific $CD8^+$ T cells (Figure 4-2B). On the other hand, 293A cells expressing the ER-targeted gB-498 minigene from WR-ESmini-gB did not prime any detectable gB-498-specific $CD8^+$ T cells. As a control, 293A cells infected with WR-Full-gB or WR-ESmini-gB elicited comparable levels of $CD8^+$ T cells against the VACV epitopes examined. A similar result was detected when 293A cells infected with the two gB-expressing MVA recombinants were used for immunisation (Figure 4-2C and D), in which full-length gB, but not the ER-targeted minigene, induced a gB-498-specific $CD8^+$ T cell response when cross presented from recombinant MVA-infected cells. In summary, the ER-targeted gB-498 minigene cannot be cross presented from WR- or MVA-infected cells to prime $CD8^+$ T cells in vivo.

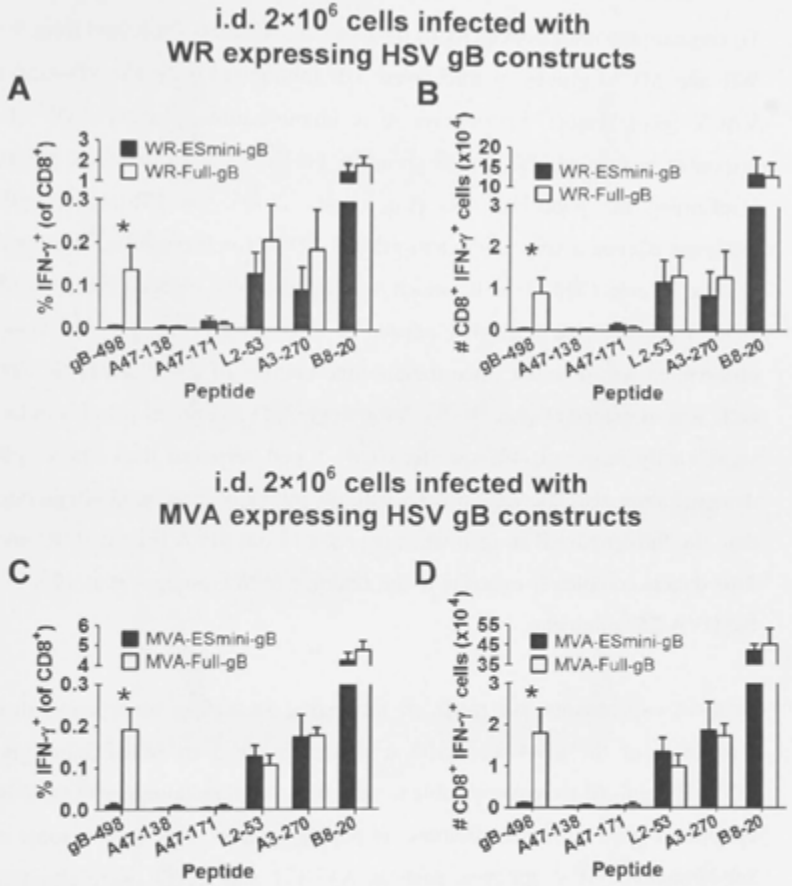


Figure 4-2| Full-length gB, but not the ER-targeted gB-498 minigene, expressed in VACV-infected cells can prime CD8$^+$ T cells in vivo via cross presentation. 293A cells were infected with the indicated recombinant WR (A and B) or MVA (C and D) at an m.o.i. of 5 for 6 h, followed by heat treatment at 60°C for 60 min. Groups of three mice were then immunised i.d. with 2×10^6 infected heat-treated cells. Seven days later, antigen-specific CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells responding to the indicated peptides are shown as mean ± SEM. Data represent results pooled from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

4.2.1.3 gB-498 co-dominates with VACV B8-20 when expressed as a minigene from VACV WR and MVA

To compare the immunogenicity of the two gB constructs expressed from VACV WR and MVA, groups of mice were i.d. immunised with the gB-expressing VACV recombinants. Seven days after immunisation, primary $CD8^+$ T cell responses against gB-498 and a group of 10 VACV epitopes were measured. Confirming the published data (Lin et al., 2013), the ER-targeted gB-498 minigene elicited a very strong anti-gB-498 $CD8^+$ T cell response, at around 7% of total splenic $CD8^+$ T cells, which was significantly higher than the 1.5% of $CD8^+$ T cells specific to gB-498 induced by WR-Full-gB (Figure 4-3A). A similar observation was detected when the absolute number of gB-498-specific $CD8^+$ T cells was measured (Figure 4-3B). Intriguingly, MVA-ESmini-gB also induced a significantly larger gB-498-specific $CD8^+$ T cell response than MVA-Full-gB, demonstrating that the ER-targeted gB-498 minigene was more immunogenic than the full-length gB antigen when expressed from MVA (Figure 4-3C and D). This data is completely opposite to the findings from Gasteiger et al. (2007) with the OVA-257 minigene.

In these experiments, we made an interesting secondary observation that the magnitude of the gB-498-specific responses induced by WR-ESmini-gB and MVA-ESmini-gB were comparable to that of the immunodominant VACV B8-20 epitope (Figure 4-3). In addition, responses against B8-20 and some other subdominant VACV epitopes, such as A47-171 and L2-53, were significantly reduced when mice were immunised with a VACV recombinant that expresses the gB-498 minigene. This immunodomination, while interesting, was not pursued further in this thesis.

In summary, the ER-targeted gB-498 minigene allows better priming of $CD8^+$ T cells than the full-length gB antigen when expressed from VACV WR and MVA. This directly correlates with the enhanced direct presentation efficiency of the ER-targeted minigene.

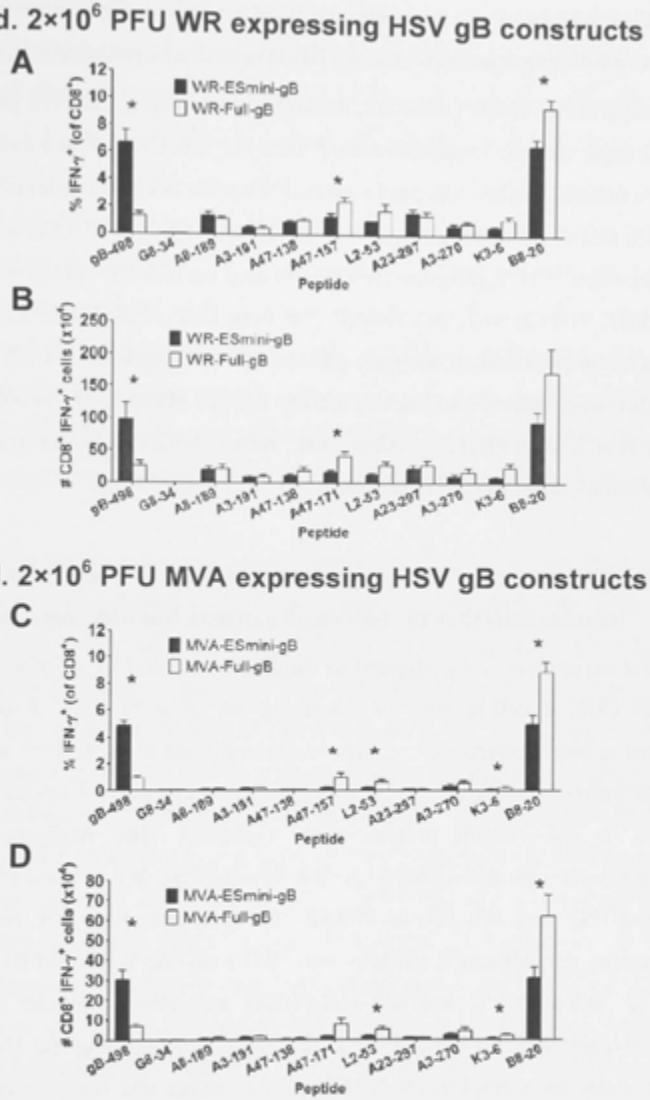


Figure 4-3| The ER-targeted gB-498 minigene is more immunogenic than the full-length gB for priming CD8$^+$ T cell responses when expressed from WR and MVA. Groups of three mice were immunised i.d. with 2×10^6 PFU of recombinant WR (A and B) or MVA (C and D) as indicated. Seven days later, percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells responding to the indicated peptides were measured by ex vivo peptide stimulation followed by ICS. Results are shown as mean ± SEM and represent data compiled from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

4.2.2 Comparison of the immunogenicity of an IAV antigen expressed either as a full-length antigen or as an ER-targeted minigene from VACV

The experiments above focused on the highly immunogenic gB-498 peptide. One could argue that the immunodominant nature of this $CD8^+$ T cell epitope is not representative of other epitopes in general. Thus we decided to identify a foreign antigen that could be recognised by a similar level of $CD8^+$ T cells as the native subdominant VACV antigens. Here we focused on five IAV antigens, including PA, PB1, PB1F2, NS2, and PR8NP (NP from IAV strain PR8). Each of these antigens contains an immunogenic peptide that is recognised by $CD8^+$ T cells in C57Bl/6 mice infected with IAV, namely PA-224 (Belz et al., 2000), PB1-703 (Belz et al., 2001), PB1F2-62 (Chen et al., 2001a), NS2-114 (Vitiello et al., 1996), and PR8NP-366 respectively (Rotzschke et al., 1990).

4.2.2.1 IAV antigens expressed as minigenes from WR are generally more immunogenic than the full-length antigens, but with some exceptions

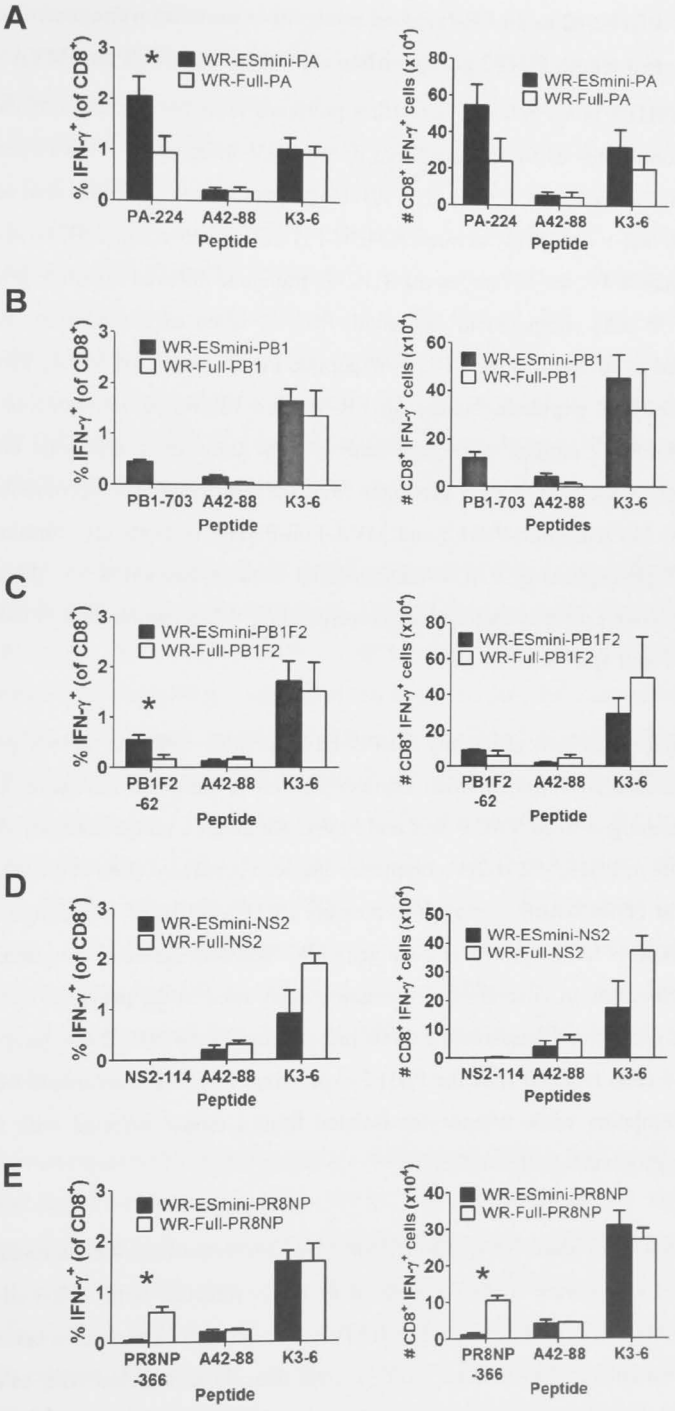
A pilot experiment was performed to identify a suitable IAV epitope as our new model $CD8^+$ T cell epitope for examining the effect of $CD8^+$ T cell priming against a less immunogenic epitope expressed from VACV. Five sets of WR recombinants, each expressing an IAV epitope either as an ER-targeted minigene or as a full- length protein, were examined. The minigene-expressing recombinants were WR-ESmini-PA, WR-ESmini-PB1, WR-ESmini-PB1F2, WR-ESmini-NS2 and WR-ESmini-PR8NP. The corresponding WR recombinants expressing the full-length antigens were WR-Full-PA, WR-Full-PB1, WR-Full-PB1F2, WR-Full-NS2 and WR-Full-PR8NP respectively. Similar to the WR recombinants used in Section 4.2.1, the transgenes encoding the IAV antigens were under the control of the VACV p7.5 promoter and were inserted into the VACV TK locus. Mice were immunised i.p. with these WR recombinants and $CD8^+$ T cell responses were measured seven days later.

We found that ER-targeted minigenes were more immunogenic than full-length antigens for three of the five IAV epitopes tested. They were PA-224, PB1-703, and PB1F2-62 (Figure 4-4A, B and C). Importantly, the responses induced by the minigenes of these three antigens were within the range of responses specific to

A42-88 and K3-6, the two subdominant VACV epitopes examined, irrespective of whether the percentages or absolute numbers of responding $CD8^+$ T cells were examined. During the statistical analysis, several observations were made. Firstly, the PB1-703-specific $CD8^+$ T cell response induced by WR-Full-PB1 was below the detection limit of this assay and so it was not possible to perform statistical analysis (Figure 4-4B). For the PB1F2-expressing viruses, there was a variation in the absolute number of $CD8^+$ T cells induced in individual mice. Therefore, although the percentage of the PB1F2-62-specific $CD8^+$ T cells induced in mice immunised with WR-ESmini-PB1F2 was significantly higher than that of WR-Full-PB1F2 (Figure 4-4C left), the difference in the total number of PB1F2-62-specific $CD8^+$ T cells was not significant (Figure 4-4C right). This was also the case for experiments with the PA-expressing viruses (Figure 4-4A right).

There were two exceptions to the observation described above. The first was NS2-114. No NS2-114-specific $CD8^+$ T cell response was detected when NS2-144 was expressed as an ER-targeted minigene or as a full-length antigen from WR (Figure 4-4D). This data suggests that NS2-114 is not immunogenic when expressed from WR in the constructs examined. This observation was not further investigated. The second was PR8NP-366. The full-length PR8NP antigen expressed from WR was immunogenic, inducing around 0.7% of $CD8^+$ T cells specific to PR8NP-366 (Figure 4-4E). However, to our surprise, the ER-targeted PR8NP-366 minigene barely primed any detectable $CD8^+$ T cell response. This contradicts the established paradigm which is that ER-targeted minigenes are at least as immunogenic as, if not more than, full-length antigens when expressed from WR (Restifo et al., 1995). This interesting phenomenon is further investigated in Chapter 5. Overall, the results here confirm the findings from the previous section using the gB498 peptide that minigenes are generally more immunogenic than full-length antigens when expressed from WR in general.

Figure 4-4| $CD8^+$ T cell epitopes from IAV expressed from WR as minigenes are generally more immunogenic than full-length antigens. Mice were immunised i.p. with 2×10^6 PFU of the WR recombinants expressing IAV $CD8^+$ T cell epitopes either as ER-targeted minigenes or as full-length antigens. The epitopes tested were PA-224 (A), PB1-703 (B), PB1F2-62 (C), NS2-114 (D) and PR8NP-366 (E). $CD8^+$ T cell responses to the peptides indicated were measured in spleens 7 days later by ex vivo peptide stimulation, followed by ICS. Percentages and absolute numbers of $CD8^+$ T cells producing IFN-γ are shown as mean ± SEM on graphs on the left and right respectively. (A) Data compiled from two independent experiments, each with groups of three mice (n = 6). (B-E) Data represent one experiment with groups of three mice. * denotes statistical significance ($p < 0.05$).


A
% IFN-γ+ (of CD8+)
WR-ESmini-PA
WR-Full-PA
PA-224
A42-88
K3-6
Peptide
CD8+ IFN-γ+ cells (x10⁴)
B
WR-ESmini-PB1
WR-Full-PB1
PB1-703
Peptide
Peptides
C
WR-ESmini-PB1F2
WR-Full-PB1F2
PB1F2 -62
D
WR-ESmini-NS2
WR-Full-NS2
NS2-114
E
WR-ESmini-PR8NP
WR-Full-PR8NP
PR8NP -366

4.2.2.2 PB1F2-62 as an ER-targeted minigene is more immunogenic than the full-length PB1F2 antigen when expressed from WR and MVA

IAV PB1F2 is a virulence factor that promotes apoptosis in IAV-infected cells and contributes to the pathogenesis during IAV infection in vivo (Chen et al., 2001a; Zamarin et al., 2006). The PB1F2-62 peptide has an amino acid sequence LSLRNPILV and binds to murine MHC-I H-2D^b (Chen et al., 2001a). As shown in Figure 4-4C, the ER-targeted PB1F2-62 minigene allowed effective priming of CD8$^+$ T cells compared to full-length PB1-F2 when expressed from WR. We decided to further test the PB1F2-62 peptide in the context of MVA. Two MVA recombinants expressing either an ER-targeted PB1F2-62 minigene or a full-length PB1F2 antigen were generated with the transgenes expressed under the control of the VACV p7.5 promoter from the TK locus. The recombinants are named MVA-ESmini-PB1F2 and MVA-Full-PB1F2 respectively. Similar to the set of gB-expressing VACV recombinants used in Section 4.2.1, these MVA recombinants directly matched the corresponding WR recombinants, WR-ESmini-PB1F2 and WR-Full-PB1F2.

We first tested how efficiently the PB1F2-62 peptide could be directly presented on infected DC2.4 cells when expressed as an ER-targeted minigene or a full-length antigen from VACV WR and MVA. Although a phage antibody fragment specific to PB1F2-62/H-2D^b complexes has been generated (Lev et al., 2008), this reagent is not sensitive enough to measure PB1F2-62/H-2D^b expression on cells that express full-length PB1F2 antigen (J.W. Yewdell, personal communication). Therefore, the in vitro direct presentation assay used in the previous section was employed again to measure the direct presentation of the PB1F2-62 epitope. Here, DC2.4 cells infected with the PB1F2-expressing VACV recombinants were used as stimulators while splenocytes isolated from a mouse infected with the IAV strain PR8 were used as effectors.

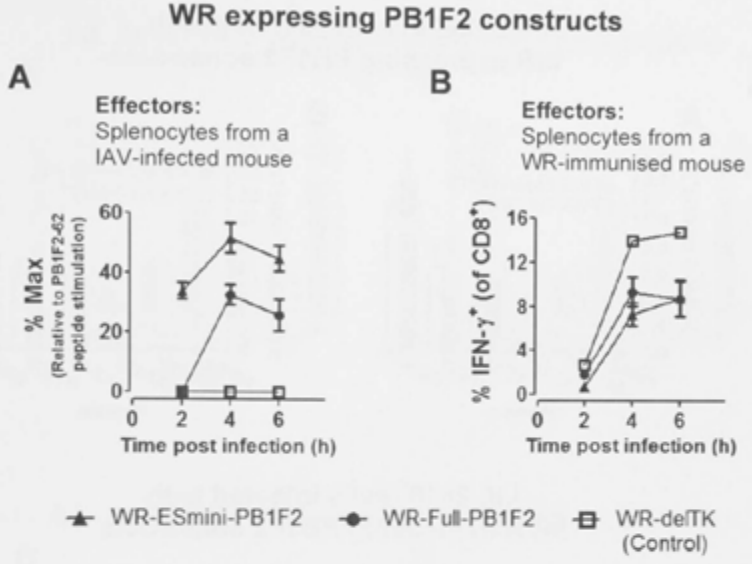
As shown in Figure 4-5A, WR-ESmini-PB1F2-infected DC2.4 cells re-stimulated more IAV-immune CD8$^+$ T cells than those infected with WR-Full-PB1F2. Similarly, DC2.4 cells infected with MVA-ESmini-PB1F2 showed a better ability to re-stimulate IAV-immune CD8$^+$ T cells than those infected with MVA-Full-PB1F2 (Figure 4-5C). Compared with the data from experiments with the WR

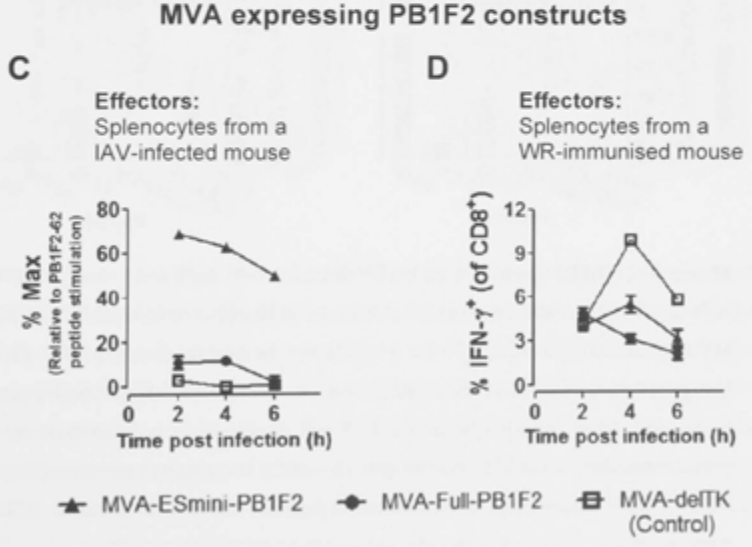
recombinants, the difference in the antigenicity between the two forms of the PB1F2 antigen seemed to be greater when expressed from MVA. However, this observation was not consistent across all independent repeats (data not shown). DC2.4 cells infected with the two control viruses, MVA-delTK and WR-delTK, failed to stimulate any $CD8^+$ T cells from the IAV-immunised mouse, suggesting that anti-IAV $CD8^+$ T cells did not recognise VACV antigens. Finally, DC2.4 cells infected with the PB1F2-expressing VACV recombinants stimulated VACV-specific $CD8^+$ T cells comparably (Figure 4-5B and D). This indicates that the difference in the level of re-stimulation of the IAV-specific $CD8^+$ T cells is not simply due to any obvious differences in the level of infection. These data show that the ER-targeted PB1F2-62 minigene allows better direct presentation than the full-length PB1F2 when expressed from VACV WR or MVA.

The cross priming ability of the PB1F2-expressing VACV recombinants from VACV infected cells were examined. Immunisation of mice with 293A cells infected with WR-ESmini-PB1F2 or WR-Full-PB1F2 induced $CD8^+$ T cell responses against multiple VACV peptides tested (Figure 4-6). However, we did not detect any cross priming of PB1F2-62-specific $CD8^+$ T cells by either the full-length PB1F2 antigen or the ER-targeted PB1F2-62 minigene available within the infected antigen donor cells. Likewise, a PB1F2-62-specific $CD8^+$ T cell response was not induced by 293A cells infected with MVA-ESmini-PB1F2 or MVA-Full-PB1F2. These results suggest that PB1F2 might not be cross presented, even when expressed as a full-length antigen from VACV-infected cells. Full-length PB1F2 antigen has been found to be unstable and has a half-life of 30 min (Chen et al., 2001a). This may contribute to its inability to prime $CD8^+$ T cells via cross presentation.

We then compared the immunogenicity of ER-targeted PB1F2-62 minigene with its full-length counterpart upon VACV immunisation. Seven days after immunising mice with the VACV recombinants expressing PB1F2 constructs via the i.d. route, the ER-targeted minigene induced a significantly larger PB1F2-62-specific $CD8^+$ T cell population than the full-length antigen, independent of the VACV strains (Figure 4-7). Around 0.4% of $CD8^+$ T cells were specific to PB1F2-62 in mice immunised with WR-ESmini-PB1F2 (Figure 4-7A). In

Figure 4-5| IAV PB1F2-62 is directly presented more effectively when expressed as an ER-targeted minigene than as a full-length antigen when expressed from VACV WR and MVA. DC2.4 cells were infected with the indicated recombinant WR (A and B) or MVA (C and D) for 5 h. They were then mixed with splenocytes from mice immunised with either IAV strain PR8 (left) or VACV WR (left) seven days earlier at a stimulator:effector ratio of 1:5. After 1 h of incubation, BFA was added, followed by another 3 h of co-culturing. ICS was then performed to measure the percentage of $CD8^+$ T cell that produced IFN-γ. Standard ICS assay with synthetic PB1F2 peptide was performed in parallel to measure the maximum possible PB1F2-62-specific T cell response. (A and C) Data show the level of PB1F2-62-specific $CD8^+$ T cell activation by the infected DC2.4 cells, after normalisation to the percentage of maximal activation induced by the synthetic PB1F2-62 peptide. (B and D) Data show the percentages of splenic $CD8^+$ T cells from a WR-immunised mouse activated by the infected DC2.4 cells. Data for the PB1F2-expressing VACV represent mean ± SEM from triplicate samples while data for the control viruses represent single measurements. Data are representative of three independent experiments.


WR expressing PB1F2 constructs
A
Effectors:
Splenocytes from a
IAV-infected mouse
% Max
(Relative to PB1F2-62
peptide stimulation)
60
40
20
0
0 2 4 6
Time post infection (h)
B
Effectors:
Splenocytes from a
WR-immunised mouse
% IFN-γ+ (of CD8+)
16
12
8
4
0
0 2 4 6
Time post infection (h)
WR-ESmini-PB1F2
WR-Full-PB1F2
WR-delTK
(Control)


MVA expressing PB1F2 constructs
C
Effectors:
Splenocytes from a
IAV-infected mouse
% Max
(Relative to PB1F2-62
peptide stimulation)
80
60
40
20
0
0 2 4 6
Time post infection (h)
D
Effectors:
Splenocytes from a
WR-immunised mouse
% IFN-γ+ (of CD8+)
12
9
6
3
0
0 2 4 6
Time post infection (h)
MVA-ESmini-PB1F2
MVA-Full-PB1F2
MVA-delTK
(Control)

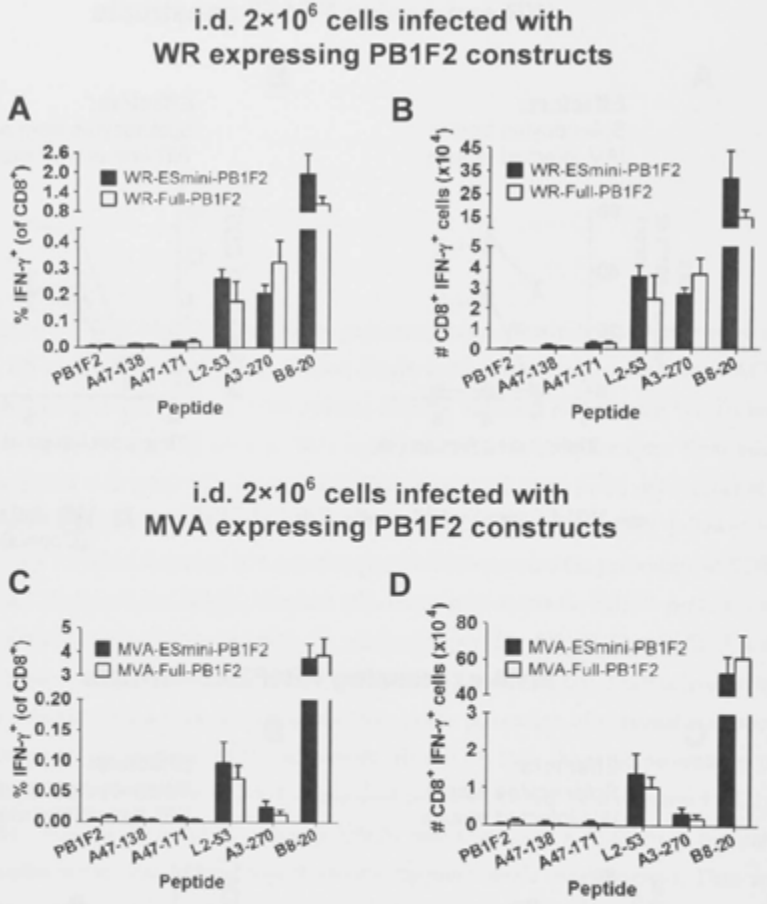


Figure 4-6| PB1F2 expressed in VACV-infected cells does not cross prime CD8$^+$ T cells in vivo. 293A cells were infected with the indicated recombinant WR (A and B) or MVA (C and D) at a m.o.i. of 5 for 6 h, followed by heat treatment at 60°C for 60 min. Groups of three mice were then immunised i.d. with 2×10^6 infected heat-treated cells. Seven days later, antigen-specific CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells responding to the indicated peptides are shown (mean ± SEM). Data represent results compiled from three independent experiments (n = 9).

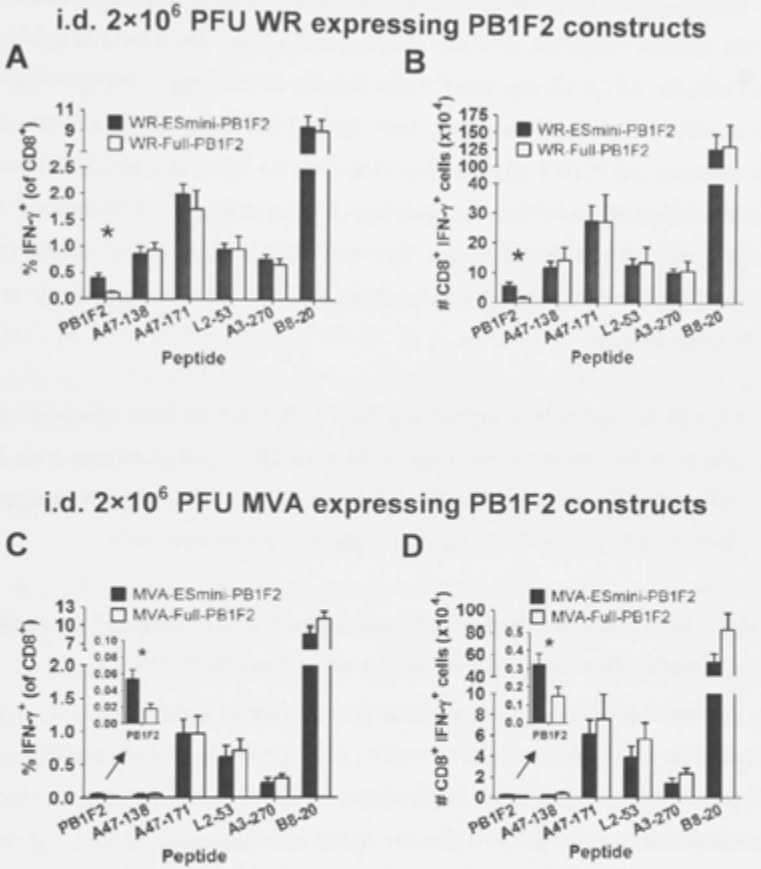


Figure 4-7| The PB1F2-62 minigene is more immunogenic than the full-length PB1F2 antigen for CD8$^+$ T cell priming when expressed from VACV WR and MVA. Groups of three mice were immunised i.d. with 2×10^6 PFU of the indicated recombinant WR (A and B) or MVA (C and D). Seven days later, percentages (A and C) and absolute numbers (B and D) of peptide-specific CD8$^+$ T cells were measured in spleens by ex vivo peptide stimulation followed by ICS. Data are shown as mean ± SEM and represent results compiled from three independent experiments (n = 9). * denotes statistical significance ($p < 0.05$).

comparison, only 0.1% of $CD8^+$ T cells recognised the PB1F2-62 peptide in mice immunised with WR-Full-PB1F2, representing a four-fold reduction in the immunogenicity. A similar difference was detected when responses were analysed as absolute numbers of $CD8^+$ T cells recognising PB1F2-62 (Figure 4-7B). Likewise, MVA-ESmini-PB1F2 was capable of inducing a stronger PB1F2-62-specific $CD8^+$ T cell response than MVA-Full-PB1F2 (Figure 4-7C and D). However, the PB1F2-62 responses elicited by the MVA recombinants were much lower than those of the corresponding WR recombinants. The inserted PB1F2 constructs are identical in the WR and MVA recombinants, so this finding suggests a role of the VACV strain in dictating the immunogenicity of some foreign antigens.

Overall, the results here suggest that PB1F2-62, a subdominant epitope from IAV, was more immunogenic when expressed as an ER-targeted minigene from VACV WR and MVA, compared to the full-length antigen. This supports our previous findings with the highly immunogenic gB-498 epitope from HSV.

4.2.3 VACV B8-20 remains immunodominant when expressed as a minigene under the control of the B8R promoter from the B8R locus

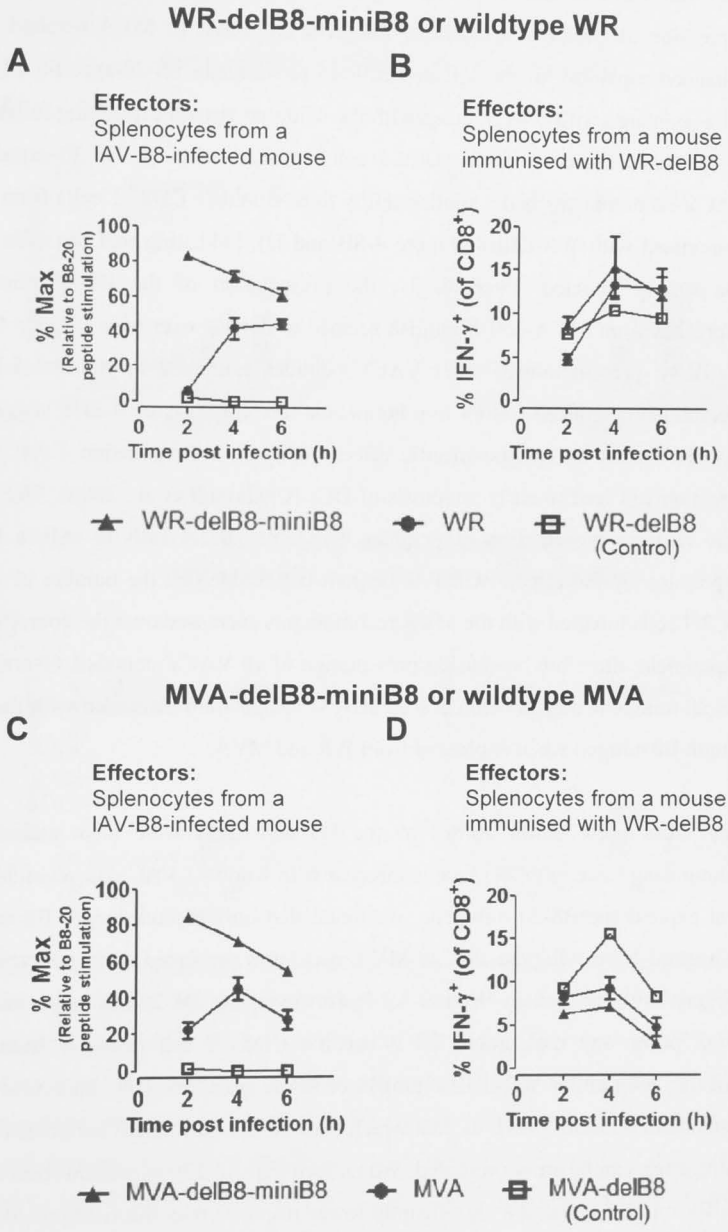
The previous two studies in this chapter demonstrate that expressing a foreign epitope as a minigene from VACV WR and MVA, which facilitates direct presentation, results in an improvement of $CD8^+$ T cell priming in vivo. This phenomenon was detected for the highly immunogenic gB-498 and the less immunogenic PB1F2-62 epitopes, suggesting that this is independent of the immunogenicity of a given antigen. However, only foreign antigens were examined. In addition, they are all expressed by the same promoter from the same insertion site (i.e. by p7.5 promoter from the VACV TK locus).

To address whether the above findings were only applicable to this specific expression setting, we examined WR and MVA recombinants, namely WR-delB8-miniB8 and MVA-delB8-miniB8, which express the VACV B8-20 epitope as a cytosolic minigene from the original B8R locus, under the control of the native B8 promoter (Wong et al., 2011; Y.C. Wong and D.C. Tscharke, unpublished data). The B8 protein expressed by WR is a soluble IFN-γ receptor

expressed from the B8R gene locus (Alcami and Smith, 1995). This protein can bind IFN-γ expressed from human and many other species effectively, but has a low affinity for murine IFN-γ (Alcami and Smith, 1995; Sroller et al., 2001). Knocking out the B8R gene from VACV WR does not affect the in vitro replication of the virus or virulence in C57Bl/6 mice (Symons et al., 2002). The amino acid sequence of the B8-20 epitope is TSYKFESV and it is restricted to MHC-I H-2K^b (Tscharke et al., 2005). The B8 antigen from MVA is truncated and non-functional (Antoine et al., 1998), but its amino-terminus is expressed and this includes the B8-20 epitope (Tscharke et al., 2005; Kremer et al., 2012). Only the cytosolic version of the B8-20 minigene was tested here because our previous results suggested that expressing B8-20 as a cytosolic or as an ER-targeted minigene from the TK locus of WR are similarly immunogenic (Y.C. Wong and D.C. Tscharke, unpublished data). The immunodominant nature of the B8-20 epitope should not affect the outcome here based on the similar findings between gB-498 and PB1F2-62 as discussed above.

To determine whether B8-20 expressed as a cytosolic minigene is directly presented more efficiently than the full-length B8 antigen, the in vitro direct presentation assay used in Figure 4-1 was employed again. Here the effectors were splenocytes from a mouse that had been infected with IAV-B8, a recombinant IAV strain PR8 expressing the VACV B8-20 peptide from the stalk region of the IAV neuraminidase protein (S.A. Smith, T. Cukalac and D.C. Tscharke, unpublished data). The CD8$^+$ T cells from this IAV-B8-immunised mouse allowed us to determine the MHC-I presentation of B8-20 on the infected cells. This is indicated by the fact that DC2.4 cells infected with VACV that do not express the B8 antigen (WR-delB8 and MVA-delB8; Symons et al., 2002; Kastenmuller et al., 2007) could not stimulate IFN-γ production in the CD8$^+$ T cells from the IAV-B8-infected mouse (Figure 4-8A and C). DC2.4 cells infected with WR-delB8-miniB8, MVA-delB8-miniB8, wildtype WR, or wildtype MVA were also tested (Figure 4-8A and C). The ability of DC2.4 infected with WR-delB8-miniB8 and MVA-delB8-miniB8 to re-stimulate B8-20-specific CD8$^+$ T cells in vitro was maximal after the first two hours of infection and it decreased over time. On the other hand, the ability of the DC2.4 cells to re-stimulate IAV-B8-immune CD8$^+$ T cells increased steadily in the first four hours of

Figure 4-8| VACV B8 expressed as a minigene is directly presented more effectively than as a full-length antigen when expressed from VACV WR and MVA. DC2.4 cells were infected with the indicated wildtype or recombinant WR (A and B) or MVA (C and D) for 5 h. They were then co-cultured with splenocytes from mice immunised with either with IAV-B8 (A and C) or WR-delB8 (B and D) seven days earlier. A stimulator:effector ratio of 1:5 was used. After 1 h of incubation, BFA was added. It was followed by another 3 h of co-culture. ICS was then performed to measure the percentage of $CD8^+$ T cells that produced IFN-γ. A standard ICS assay with a synthetic B8-20 peptide was performed in parallel with splenocytes from the IAV-B8-immunised mouse to measure the total B8-20-specific $CD8^+$ T cell response. (A and C) Data show the level of B8-20-specific $CD8^+$ T cell activation by the infected DC2.4 cells, after standardisation to the percentage of maximal potential activation induced by the synthetic B8-20 peptide. (B and D) Data show the percentages of splenic $CD8^+$ T cells from a WR-delB8-immunised mouse that were activated by the infected DC2.4 cells. Data for the B8-expressing VACV represent mean ± SEM from triplicate samples while data for the control viruses represent single measurements. Data are representative of five independent experiments.


WR-delB8-miniB8 or wildtype WR
A
Effectors:
Splenocytes from a
IAV-B8-infected mouse
% Max
(Relative to B8-20
peptide stimulation)
Time post infection (h)
B
Effectors:
Splenocytes from a mouse
immunised with WR-delB8
% IFN-γ+ (of CD8+)
Time post infection (h)
WR-delB8-miniB8
WR
WR-delB8
(Control)
MVA-delB8-miniB8 or wildtype MVA
C
Effectors:
Splenocytes from a
IAV-B8-infected mouse
% Max
(Relative to B8-20
peptide stimulation)
Time post infection (h)
D
Effectors:
Splenocytes from a mouse
immunised with WR-delB8
% IFN-γ+ (of CD8+)
Time post infection (h)
MVA-delB8-miniB8
MVA
MVA-delB8
(Control)

infection with wildtype WR and wildtype MVA. It plateaued for cells infected with WR while it decreased when cells were infected with MVA. Importantly, expression of B8-20 as a cytosolic minigene from WR or MVA resulted in an enhanced capability of the infected cells to re-stimulate B8-20-specific $CD8^+$ T cells compared with cells infected with the wildtype viruses expressing full-length B8 proteins. By comparison, DC2.4 cells infected with the B8-20-expressing VACV recombinants had a similar ability to re-stimulate $CD8^+$ T cells from mice immunised with WR-delB8 (Figure 4-8B and D), indicating that the cells were comparably infected. Interestingly, the presentation of the B8-20 minigene expressed from MVA-delB8-miniB8 seemed to decline over time (Figure 4-8C). Similarly, presentation of other VACV peptides expressed by the tested MVA recombinants reduced from 4 to 6 hours post infection (Figure 4-8D), suggesting that this is not an epitope-specific effect. As discussed in section 1.9.4, MVA infection can lead to early apoptosis of DCs (Chahroudi et al., 2006). The assay here requires presentation of peptides on viable DC2.4 cells to induce IFN-γ expression by the effector $CD8^+$ T cells. It is possible that the number of viable DC2.4 cells infected with the MVA recombinants decreased over the course of the experiment, therefore decreasing presentation of all VACV peptides. Overall, the B8-20 minigene allows better B8-20/MHC-I presentation compared with the full-length B8 antigen when expressed from WR and MVA.

The cross presentation ability of the B8 constructs were also studied. By immunising mice with 293A cells infected with wildtype VACV or recombinants that express the B8-20 minigene, we found that only the full-length B8 antigen expressed from wildtype WR or MVA was cross presented from infected cells (Figure 4-9), as seen in Section 3.2.3. However, the B8-20 minigene failed to cross prime any measurable B8-20-specific $CD8^+$ T cell response from cells infected with either WR-delB8-miniB8 or MVA-delB8-miniB8. This result is in agreement with our previous data which showed that full-length antigens, but not minigenes, can be cross presented. We noticed that A3-270 expressed from MVA-delB8-miniB8 induced a significantly lower response than the wildtype MVA in this cross priming assay (Figure 4-9C and D). However, this did not occur for the L2-53-specific response, suggesting that the antigen donor cells were indeed infected with the recombinant virus to allow VACV antigen expression. In

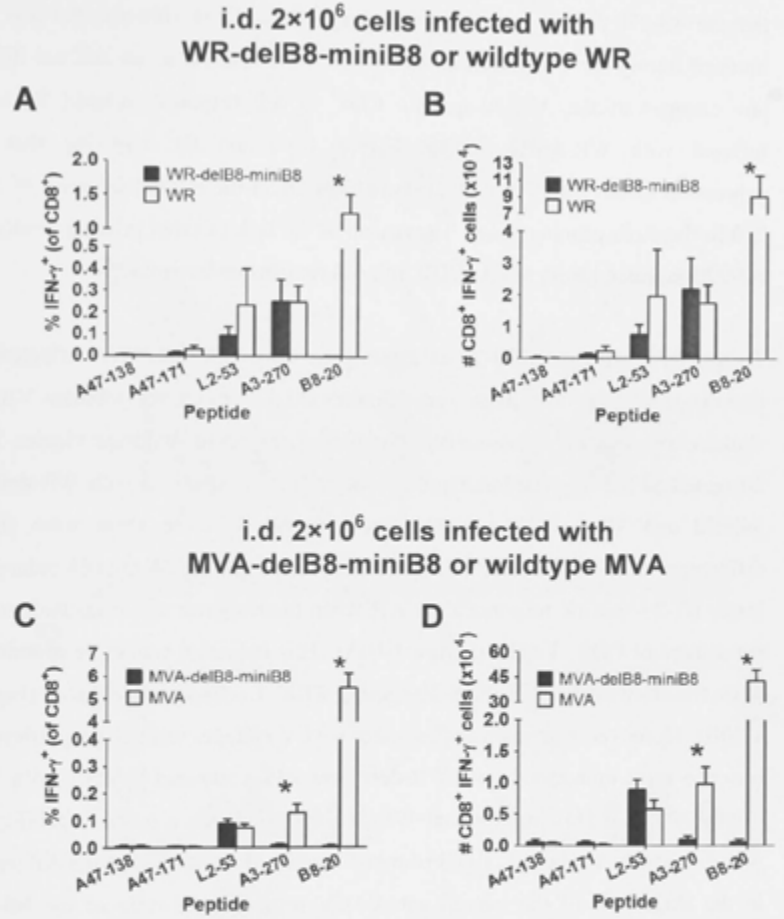


Figure 4-9| Full-length B8, but not the B8-20 minigene, expressed in VACV-infected cells cross primes B8-20-specific CD8$^+$ T cells. 293A cells were infected with the indicated recombinant WR (A and B) or MVA (C and D) at an m.o.i. of 5 for 6 h, followed by heat treatment as shown in Figure 4-1. Groups of three mice were then i.d. immunised with 2×10^6 of these heat-treated cells. Seven days later, antigen-specific CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells responding to the indicated peptides are shown. Data represent results compiled from two independent experiments (mean ± SEM; n = 6). * denotes statistical significance ($p < 0.05$).

addition, a $CD8^+$ T cell response against A3-270 was detected in mice immunised directly with MVA-delB8-miniB8 (refer to Figure 4-10D and E). This indicates that the A3-270 peptide can be generated and presented in vivo and that it is not mutated during the generation of MVA-delB8-miniB8. Also, we did not detect any changes of the A3-270-specific $CD8^+$ T cell response induced by cells infected with WR-delB8-miniB8 (Figure 4-9A and B), implying that an expression of the B8-20 minigene should not affect the immunogenicity of A3-270 in the cross priming assay. The reason of the lack of cross priming ability of A3-270 expressed from MVA-delB8-miniB8 remains undetermined.

To test how expressing B8-20 as a minigene from WR and MVA affected its immunogenicity in vivo, mice were immunised i.d. with the recombinant VACV strains expressing the cytosolic B8-20 minigene or with the wildtype viruses. B8-20 remained the immunodominant epitope in mice immunised with WR-delB8-miniB8 and MVA-delB8-miniB8 (Figure 4-10). However, there were some differences between the results from WR and MVA. WR-delB8-miniB8 induced a lower B8-20-specific response than WR when the response was measured as the percentage of $CD8^+$ T cells (Figure 4-10A). This reduction was more prominent when the absolute number of B8-20-specific $CD8^+$ T cells was determined (Figure 4-10B). Moreover, responses against other VACV epitopes were also significantly lower in mice immunised with WR-delB8-miniB8, compared to those with WR (Figure 4-10B). This implies that WR-delB8-miniB8 has a lower capability to prime an anti-VACV $CD8^+$ T cell response in general. To normalise the difference in the magnitude of the overall anti-VACV response, the ratio of the B8-20-specific response to the sum of responses specific for all VACV epitopes was examined (Figure 4-10C). From this analysis, we found that WR-delB8-miniB8 and WR both induced a similar level of anti-B8-20 response relative to the overall anti-VACV responses (around 60%). For MVA, expression of B8-20 as a cytosolic minigene enhanced its immunogenicity, as measured by the percentage of $CD8^+$ T cells or by the absolute number of B8-20-specific $CD8^+$ T cells (Figure 4-10D and E). In contrast to WR-delB8-miniB8, we did not detect any reduction of the numbers of VACV-specific $CD8^+$ T cells induced by MVA-delB8-miniB8. This suggests that expression of the B8-20 minigene does not affect the level of anti-VACV $CD8^+$ T cell priming in general. We found that MVA-delB8-miniB8

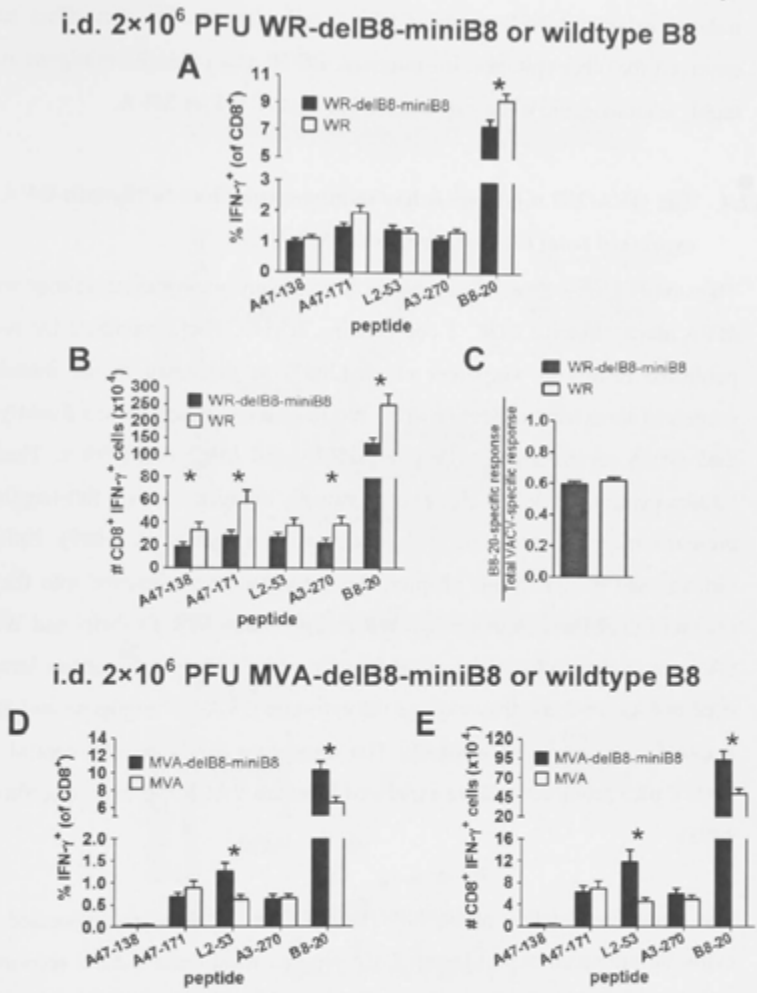


Figure 4-10| Despite some variations in the B8-20-specific response induced, B8-20 epitope remains immunodominant when expressed as a minigene from VACV WR and MVA. Groups of three mice were immunised i.d. with 2×10^6 PFU of recombinant WR (A and B) or MVA (D and E) as indicated. Seven days later, the percentage (A and D) and absolute number (B and E) of $CD8^+$ T cells responding to the indicated peptides were measured by ex vivo peptide stimulation followed by ICS. (C) The level of anti-B8-20 $CD8^+$ T cell responses induced by the two WR recombinants relative to sums of responses induced against all VACV epitopes examined is shown. The result was calculated based on data from (B). (A, B, D and E) Data represent results compiled from five independent experiments (mean ± SEM; n = 15). * denotes statistical significance ($p < 0.05$).

induced a larger L2-53-specific $CD8^+$ T cell response, but this effect was not observed for other epitopes. In summary, B8-20 as a cytosolic minigene remains highly immunogenic when expressed from VACV WR or MVA.

4.2.4 The OVA-257 minigene is less immunogenic than full-length OVA when expressed from the delIII region of MVA

The studies above clearly demonstrate that antigens expressed as minigenes from MVA allow effective $CD8^+$ T cell priming in vivo. This contradicts the previous published results by Gasteiger et al. (2007) as discussed in the introduction section of this chapter (Section 4.1). We re-examined their claims directly using their MVA recombinants, MVA-delIII-SIIN and MVA-delIII-OVA. These two viruses express OVA-257 either as a cytosolic minigene or as a full-length OVA protein under the control of p7.5 promoter (Gasteiger et al., 2007). Unlike the viruses used earlier in this chapter, the transgenes were inserted into the delIII region of the MVA genome. Two WR recombinants, WR-TK-SIIN and WR-TK-OVA, were also included here (Restifo et al., 1995). These two viruses have been used in Chapter 3 and they express the cytosolic OVA-257 minigene and the full-length OVA protein respectively. The transgenes are under the control of the VACV p7.5 promoter and are expressed from the VACV TK locus (Restifo et al., 1995).

The direct presentation ability of OVA-257 on $H\text{-}2K^b$ when expressed as the cytosolic minigene or full-length OVA antigen from these VACV recombinants was measured directly from infected DC2.4 cells with the 25D1.16 antibody. The expression of the OVA-257 minigene in the DC2.4 cells infected with WR-TK-SIIN or MVA-delIII-SIIN allowed better OVA-257/MHC-I presentation on the cell surface than those infected with WR-TK-OVA and MVA-delIII-OVA respectively, over the period of 6 hours of infection (Figure 4-11). The presentation level of OVA-257 when expressed as a cytosolic minigene from WR and MVA reached its maximum level at around 4 hours post infection. In comparison, presentation of OVA-257 from the full-length OVA antigen increased more slowly over time until 4 hours post-infection, but the presentation level of OVA-257 remained much lower than that of the OVA-257 minigene. Our

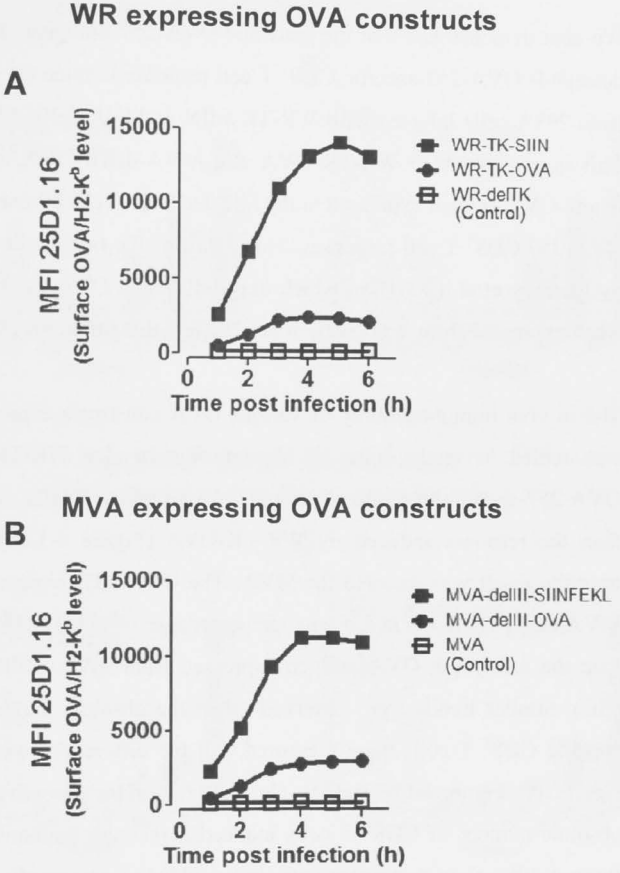


Figure 4-11| The cytosolic OVA-257 minigene is better at direct presentation than the full-length OVA antigen when expressed from the TK locus of VACV WR or from the delIII region of MVA. DC2.4 cells were infected with the indicated wildtype or recombinant WR (A) or MVA (B) at an m.o.i. of 5. At times indicated, aliquots of infected cells were collected and labelled with APC-conjugated 25D1.16 antibody prior to flow cytometric analysis to measure the mean fluorescence intensity of APC of the labelled cells. Data are representative of four independent experiments.

results here confirm the previous studies using these recombinants (Porgador et al., 1997; Princiotta et al., 2003; Gasteiger et al., 2007).

We next demonstrated that the cytosolic OVA-257 minigene did not induce any detectable OVA-257-specific $CD8^+$ T cell response in mice via cross presentation from 293A cells infected with WR-TK-SIIN or MVA-delIII-SIIN (Figure 4-12). This is in contrast to WR-TK-OVA and MVA-delIII-OVA, in which the full-length OVA antigen expressed in the infected 293A cells allowed priming of anti-OVA-257 $CD8^+$ T cell responses. This confirms the findings in Section 3.2.2 and by Norbury et al. (2004), in which only full-length OVA can be cross presented when expressed from WR (Section 3.2.2). Here this finding is extended to MVA.

The in vivo immunogenicity of various OVA constructs expressed from VACV was studied. Seven days after i.d. immunisation in mice, WR-TK-SIIN induced an OVA-257-specific response of around 2.5% of $CD8^+$ T cells, significantly higher than the response induced by WR-TK-OVA (Figure 4-13A). In contrast, the opposite result was detected for MVA. The OVA-257 minigene expressed from MVA-delIII-OVA elicited a smaller percentage of anti-OVA-257 $CD8^+$ T cells than the full-length OVA antigen expressed from MVA-delIII-OVA (Figure 4-13C). Similar trends were observed when the absolute numbers of OVA-257-specific $CD8^+$ T cells were measured, but the differences were not statistically significant (Figure 4-13B and D). The main reason for this was the variation in the absolute number of $CD8^+$ T cells induced across the immunised mice. Overall, these results are in agreement with those published previously using these OVA-expressing recombinants (Restifo et al., 1995; Gasteiger et al., 2007). However, the lower immunogenicity of the OVA-257 minigene expressed from MVA disagrees with our previous results with MVA recombinants expressing gB-498, PB1F2-62 and B8-20.

4.2.5 The insertion site in the MVA genome influences the relative immunogenicity of the OVA-257 minigene compared to full-length OVA

The major difference between the MVA recombinants generated in this chapter and those in Gasteiger et al. (2007) is the insertion site of the transgenes encoding the foreign antigens within the MVA genome. Therefore, we examined whether

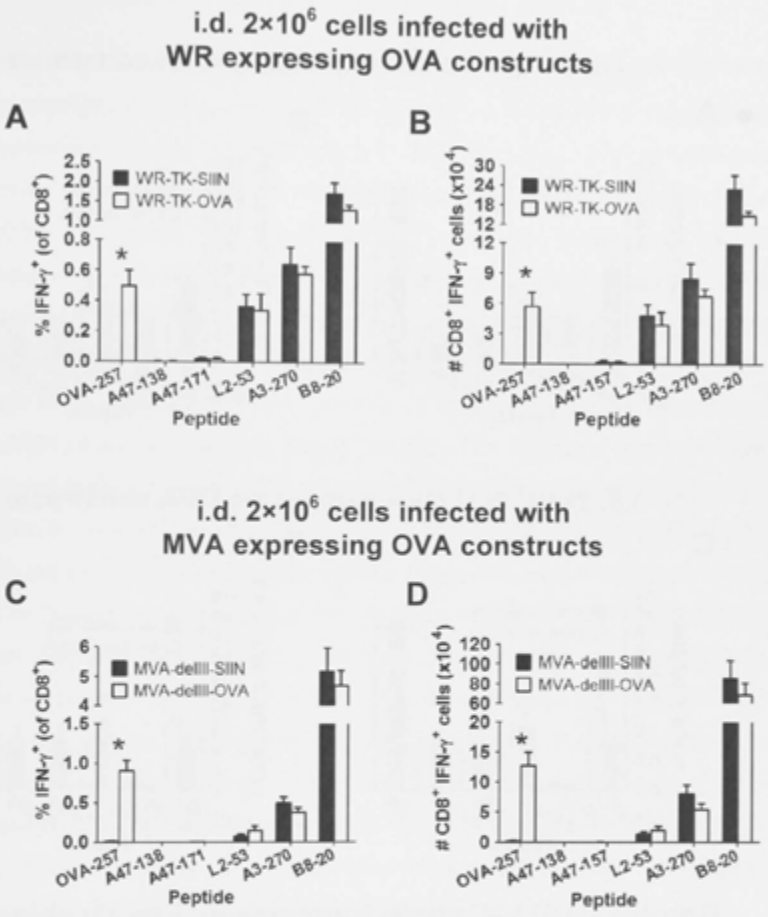


Figure 4-12| OVA-257-specific CD8$^+$ T cells cannot be cross primed by the OVA-257 minigene expressed within VACV-infected cells. 293A cells were infected with the indicated recombinant WR (A and B) or MVA (C and D) for 6 h, followed by heat treatment as described in Figure 4-2. Mice were then immunised i.d. with 2×10^6 of these infected, heat-treated cells. Seven days later, peptide-specific CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells responding to the indicated peptides were shown. Data represent results compiled from groups of three mice from two independent experiments (n = 6). * denotes statistical significance ($p < 0.05$).

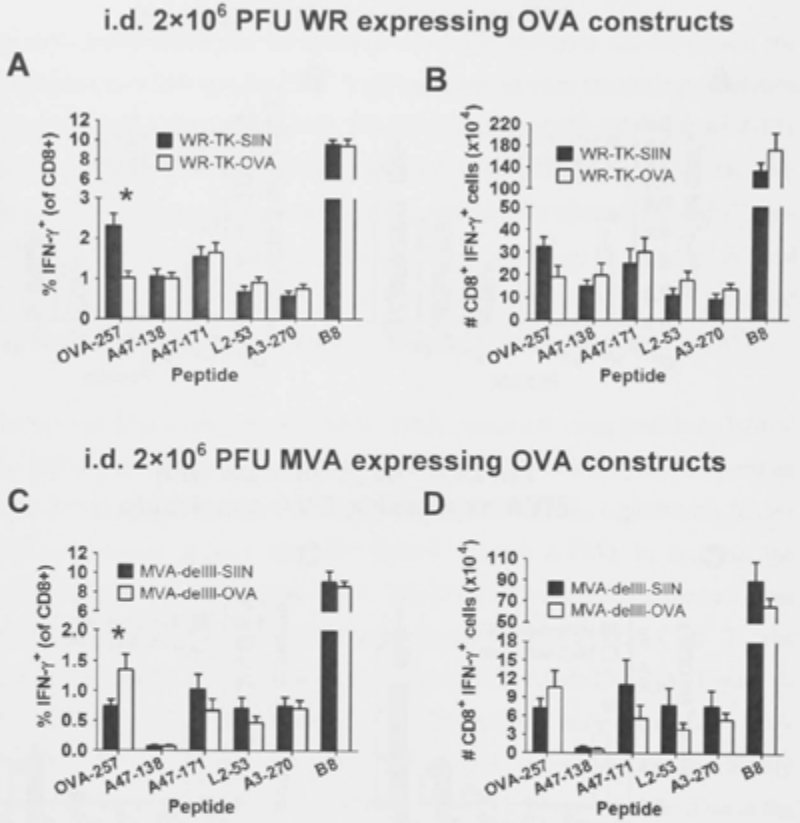


Figure 4-13| The OVA-257 minigene is more immunogenic than the full-length OVA when expressed from the TK locus of WR while the opposite result was observed when the OVA constructs were expressed from the delIII region of MVA. Groups of three mice were immunised i.d. with 2×10^6 PFU of recombinant WR (A and B) or MVA (C and D) as indicated. Seven days later, percentages (A and C) and absolute numbers (B and D) of $CD8^+$ T cells responding to the indicated peptides were measured in spleens by ex vivo peptide stimulation followed by ICS. Data represent results compiled from three independent experiments (n= 9). * denotes statistical significance ($p < 0.05$).

the choice of insertion site would affect the immunogenicity of the OVA-257 minigene relative to the full-length OVA antigen.

Firstly, two MVA recombinants, namely MVA-TK-SIIN and MVA-TK-OVA, were examined. The first MVA recombinant has been introduced in Chapter 3 and it expresses a cytosolic OVA-257 minigene under the control of the p7.5 promoter from the MVA TK locus. As shown in Chapter 3, this virus can effectively prime an OVA-257-specific $CD8^+$ T cell response in vivo. Here this virus was directly compared with MVA-TK-OVA, a MVA recombinant expressing full-length OVA by the p7.5 promoter inserted into the MVA TK locus. The OVA-257 minigene expressed by MVA-TK-SIIN is directly presented more effectively than the full-length OVA expressed by MVA-TK-OVA on infected DC2.4 cells as measured by 25D1.16 antibody staining (Figure 4-14A). This finding is similar to those observed in Figure 4-11B which used the MVA recombinants that express OVA constructs from the deletion III region. The in vivo immunogenicity of MVA-TK-SIIN and MVA-TK-OVA was then studied. When mice were immunised i.d. with MVA-TK-SIIN, around 2.5% of $CD8^+$ T cells specific to OVA-257 were elicited seven days later (Figure 4-14B). This is significantly higher than that found in mice immunised with MVA-TK-OVA. When the absolute numbers of anti-OVA-247 $CD8^+$ T cells were compared, a similar trend was observed although the difference was not significant (Figure 4-14C). These data from this set of MVA recombinants, which directly matched the WR recombinants used in Figure 4.13, demonstrate that OVA-257 as a cytosolic minigene can be more immunogenic than a full-length OVA when expressed from MVA.

Next, another set of MVA recombinants were examined. They express the cytosolic OVA-257 minigene or the full-length OVA antigen under the control of the VACV p7.5 promoter from the intergenic region between two essential genes, A11R and A12L (Wong et al., 2011). These two viruses are called MVA-A11/A12-SIIN and MVA-A11/A12-OVA respectively. Similar to the previous in vitro studies with other sets of OVA-expressing recombinants, direct presentation of OVA-257 on the surface of infected DC2.4 cells was enhanced when the epitope was expressed as a minigene from MVA-A11/A12-SIIN compared to the full-length OVA expressed from MVA-A11/A12-OVA (Figure 4-15A). Despite

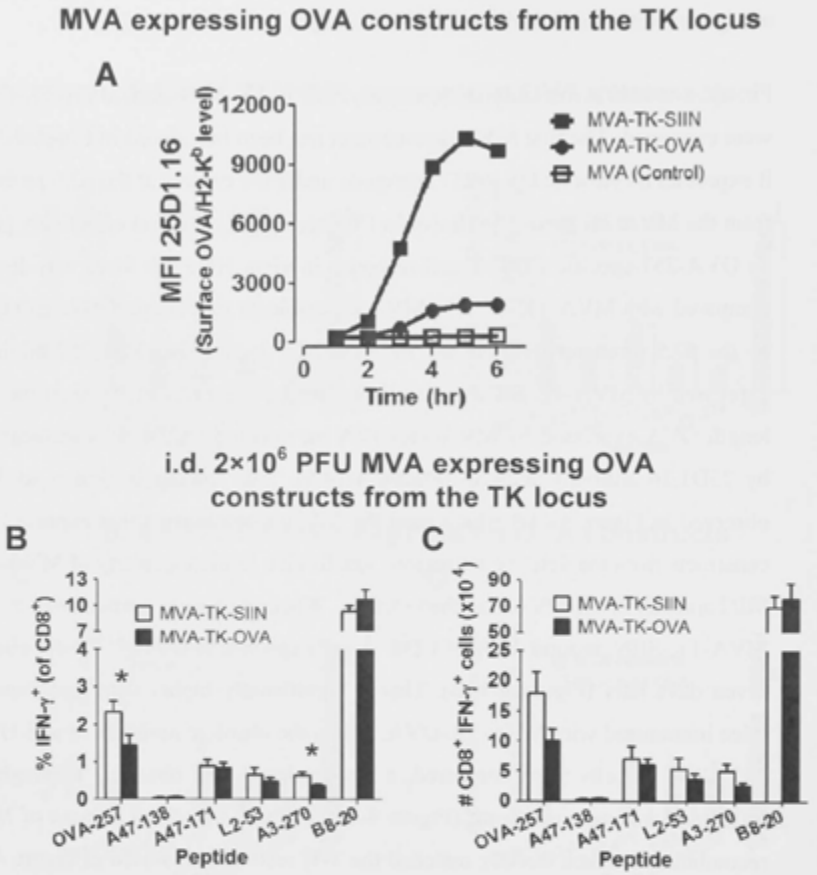


Figure 4-14| The OVA-257 minigene is more immunogenic than the full-length OVA when expressed from the TK locus of MVA. (A) DC2.4 cells were infected with the indicated wildtype or recombinant MVA. At times indicated, aliquots of infected cells were collected and labelled with APC-conjugated 25D1.16 antibody prior to flow cytometric analysis to measure the mean fluorescence intensity of APC of the labelled cells. (B and C) Groups of three mice were immunised i.d. with 2×10^6 PFU of MVA-TK-SIIN or MVA-TK-OVA. Seven days later, percentages (B) and absolute numbers (C) of splenic $CD8^+$ T cells responding to the indicated peptides were measured by ex vivo peptide stimulation followed by ICS. (A) Data are representative of five independent experiments. (B and C) Data are compiled from three independent experiments (n = 9). * denotes statistical significance ($p < 0.05$).

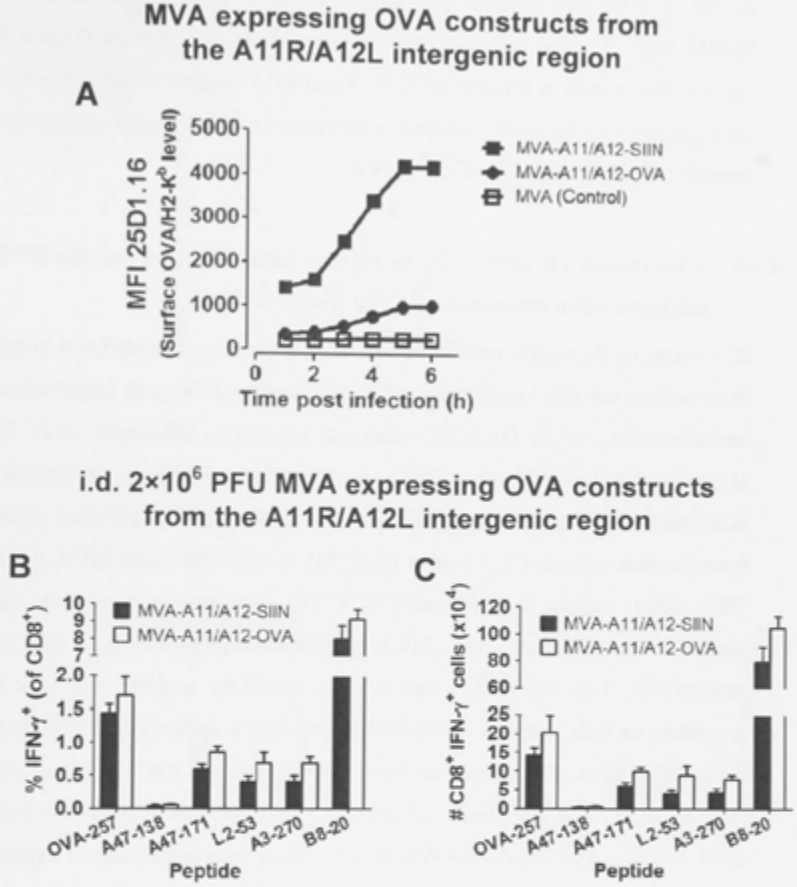


Figure 4-15| The OVA-257 minigene and the full-length OVA are similarly immunogenic when expressed from the A11/A12 intergenic region of MVA. (A) DC2.4 cells were infected with the MVA as indicated. Aliquots of infected cells were collected every hour for six hours and were labelled with APC-conjugated 25D1.16 antibody prior to flow cytometric analysis to measure the MFI of APC on the labelled cells. (B and C) Groups of 3-4 mice were immunised i.d. with 2×10^6 PFU of MVA-A11/A12-SIIN or MVA-A11/A12-SIIN. Seven days later, peptide-specific CD8$^+$ T cell responses were measured by ex vivo peptide stimulation followed by ICS. Results are shown as percentages (B) and absolute numbers (C) of responding CD8$^+$ T cells. (A) Data are representative of three independent experiments. (B and C) Data are compiled from four independent experiments (n = 13).

this in vitro difference, these two viruses induced similar levels of OVA-257-specific $CD8^+$ T cell response in mice immunised via the i.d. route (Figure 4-15B and C). The results in Figures 4-13, 4-14 and 4-15 suggest that the choice of an insertion site can determine whether a minigene or a full-length antigen is more immunogenic when expressed from MVA.

4.2.6 A functional TK influences the relative immunogenicity of the OVA-257 minigene when compared with full-length OVA

By comparing the results with the MVA recombinants expressing OVA constructs from various separated loci, it seemed that a functional TK gene might reduce the immunogenicity of the OVA-257 minigene, relative to full-length OVA. This is because MVA-TK-SIIN, which has a disrupted TK gene due to the foreign gene insertion, was more immunogenic than the full-length OVA antigen expressed from the same region. On the other hand, MVA-delIII-SIIN and MVA-A11/A12-SIIN, which express a functional VACV TK, were less immunogenic than or similarly immunogenic as MVA-delIII-OVA and MVA-A11/A12-OVA respectively. This hypothesis was directly tested by making two new MVA recombinants based on MVA-delIII-SIIN and MVA-delIII-OVA from Gasteiger et al. (2007). An empty construct from transfer plasmid pSC11GB was inserted into the TK locus in the genome of these two recombinants to create MVA-delIII-SIIN-delTK and MVA-delIII-OVA-delTK. These two recombinants express the OVA-257 minigene or full-length OVA from the delIII region respectively, but do not express functional TK.

DC2.4 cells infected with MVA-delIII-SIIN-delTK showed a more effective OVA-257/H-$2K^b$ presentation on the cell surface than those infected with MVA-delIII-OVA-delTK (Figure 4-16A). This indicated that deletion of the VACV TK gene does not alter the enhanced ability of the OVA-257 minigene in direct presentation. When mice were immunised with these two viruses, a similar level of anti-OVA-257 $CD8^+$ T cell responses were induced (Figure 4-16B and C). This result was in between what we observed with the MVA recombinants expressing OVA constructs from delIII region (refer to Figure 4-13) and those expressing OVA constructs from VACV TK locus (refer to Figure 4-14). As a side note,

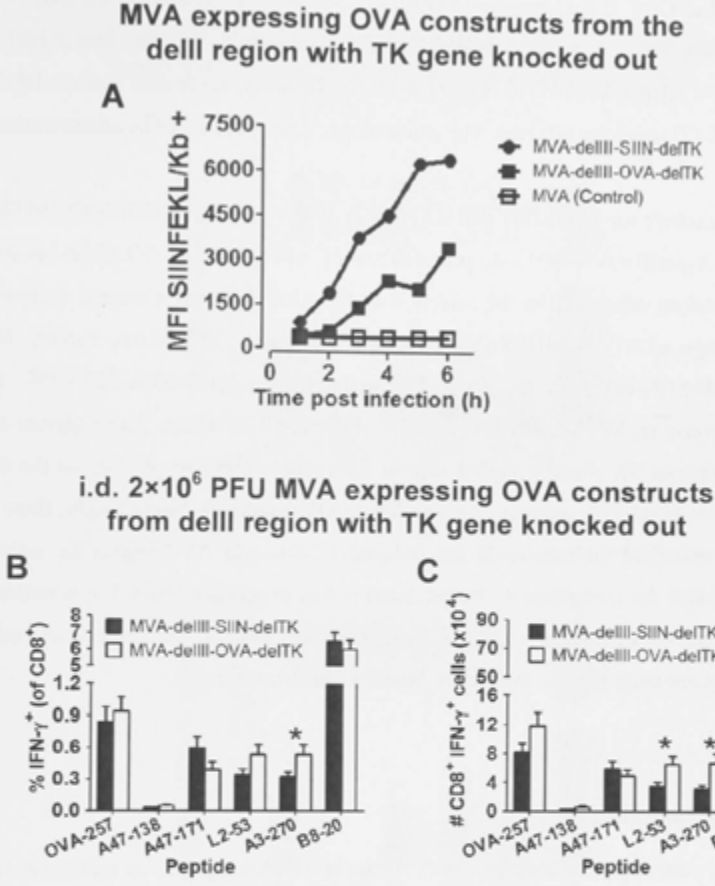


Figure 4-16| The OVA minigene expressed from delIII region of MVA becomes similarly immunogenic as the full-length OVA when the VACV TK gene is knocked out. (A) DC2.4 cells were infected with the indicated wildtype MVA or MVA recombinants. At time indicated, aliquots of infected cells were collected and labelled with APC-conjugated 25D1.16 antibody. MFI of APC on the labelled cells was determined by flow cytometric analysis. (B-C) Groups of three mice were immunised i.d. with 2×10^6 PFU of the MVA-delIII-SIIN-delTK or MVA-delIII-OVA-delTK. Seven days after immunisation, splenocytes from the immunised mice were stimulated with the indicated peptides ex vivo, followed by ICS. Results are shown as percentages (B) or absolute numbers (C) of the responding $CD8^+$ T cells. (A) Data are representative of three independent experiments. (B and C) Data are compiled from five independent experiments (n = 15). * denotes statistical significance ($p < 0.05$).

smaller $CD8^+$ T cell responses against L2-53 and A3-270 were induced in mice immunised with MVA-delIII-SIIN-delTK compared to those immunised with MVA-delIII-OVA-delTK (Figure 4-16C). However, responses against B8-20 or A47-171 were not affected. The cause of this difference remains undetermined.

To exclude the possibility that the similar ability of MVA-delIII-SIIN-delTK and MVA-delIII-OVA-delTK to prime OVA-257-specific $CD8^+$ T cells is because of a random effect during the selection of the recombinants, a second independent lineage of MVA-delIII-SIIN-delTK was examined. This virus, namely MVA-delIII-SIIN-delTK-2, also elicited a similar level of anti-OVA-257 $CD8^+$ T cell response as MVA-delIII-OVA-delTK (Figure 4-17). These data suggests that a functional TK protein plays a role in the reduced immunogenicity of the OVA-257 minigene compared to the full-length OVA antigen. Interestingly, there were no statistical differences in the induced L2-54- and A3-270-specific responses amongst the recombinant viruses tested in this experiment (which was detected in Figure 4-16C). This suggests variations of $CD8^+$ T cell responses induced between experiments, even with the use of identical viruses.

4.3 Discussion

The studies in this chapter clearly demonstrate that peptides as minigenes can be more immunogenic than full-length antigens when expressed from MVA, but there are some exceptions. Importantly, the choice of an insertion site influences the immunogenicity of a given antigen expressed as a minigene from MVA. Further, these data suggest that WR and MVA expressing foreign antigens from the TK locus allow effective direct priming. One study has demonstrated that expressing human cytomegalovirus proteins as rapidly-degraded constructs from the deletion II region of MVA could induce $CD8^+$ T cell responses against the antigens tested in mice (Wang et al., 2004). It should be noted that $CD8^+$ T cell responses were measured using a cytotoxicity assay after in vitro $CD8^+$ T cell expansion (Wang et al., 2004). Despite some limitations, the study by Wang et al. (2004) and the studies shown in this chapter demonstrate that $CD8^+$ T cell responses against antigens expressed from MVA can be induced via direct

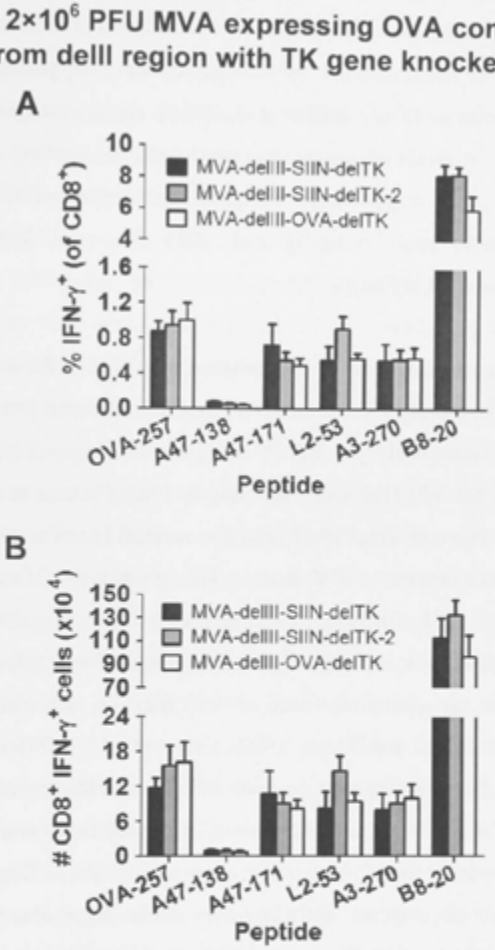


Figure 4-17| A second independent lineage of MVA-delIII-SIIN-delTK confirms that a functional VACV TK protein influences the immunogenicity of the OVA-257 minigene expressed from the delIII region. Groups of three mice were i.d. immunised with 2×10^6 PFU of the recombinant MVA indicated. After seven days, percentages (A) and absolute numbers (B) of $CD8^+$ T cells responding to the indicated peptides were determined with ex vivo peptide stimulation, followed by ICS. Data represent results from three independent experiments (n = 9).

presentation effectively and are not dissimilar from WR in general. This finding is further supported by the observation that MVA-infected DCs interact with naive $CD8^+$ T cells in the draining LNs in infected mice, suggesting a role of direct $CD8^+$ T cell priming (Kastenmüller et al., 2013). As an extension of our findings, it has been reported that minigenes expressed from avipoxvirus, another species of poxvirus that does not replicate in mammals, could prime $CD8^+$ T cell responses in mice and in humans (Rosenberg et al., 2003; Tine et al., 2005). These reports are further discussed in Chapter 7.

So far, studies about the relationship between the effect of the insertion site within the VACV genome and the immunogenicity of the inserted foreign antigens have been limited. Historically, the VACV TK gene has been used as one of the first insertion sites for WR (Panicali and Paoletti, 1982; Flexner et al., 1987). This is because there is a well-established selection method based on the use of BUdR to select against wildtype VACV that is TK^+ phenotype (Mackett et al., 1982; Mackett et al., 1984; Chakrabarti et al., 1985). Other insertion sites have been used, but they are less common. On the other hand, large deletion regions in the MVA genome are commonly used as the insertion site for generating MVA recombinants (Sutter and Moss, 1992; Sutter et al., 1995). A previous study reported that it was difficult to generate MVA recombinants expressing antigens from the MVA TK locus, and the authors suggested that knocking out TK from MVA may reduce its in vitro replication ability (Scheiflinger et al., 1996). However, multiple reports, including this study, have successfully generated recombinant MVA using TK as the insertion site and some have been used in human clinical trials (Sanchez-Puig et al., 2004, Kovacs et al., 2003, Cottingham et al., 2008, Hanke and McMichael, 2000, Létourneau et al., 2007, Hanke et al., 1998). These support the idea that MVA carrying transgenes inserted in the VACV TK locus are indeed viable. Further, our study here implies that this locus may be a better insertion site for minigene constructs.

Studies in this chapter indicate a role of insertion site on determining the immunogenicity of a given minigene construct when expressed from MVA. One obvious explanation is that the expression level of a transgene might be different when expressed from different insertion sites. Several studies have examined if

the insertion site influences the expression level of the transgenes from VACV. Expression of a murine MHC-I heavy chain H-2K^d gene inserted into the VACV TK locus of VACV WR was comparable to that expressed from the *Hind*III F insertion site, a less common insertion site used in constructing WR recombinants (Coupar et al., 1988). In another study, three independent WR recombinants were generated, each expressing the human IL-2 gene from a different insertion loci, namely the *Hind*III C region, VACV TK locus or VACV haemagglutinin locus (Flexner et al., 1987). All three recombinants were attenuated in mice to a similar level due to the IL-2 expression (Flexner et al., 1987). Kunke et al. (1993) generated a recombinant VACV using strain Praha by inserting the surface and core antigens from hepatitis B virus into the VACV TK and the K1L (a host range gene) loci respectively. The investigators found that the expression level of these two antigens was not influenced if their insertion sites in the VACV genome were switched (Kunke et al., 1993). A separate study demonstrated that insertion of foreign genes into VACV TK locus allow higher transgene expression than genes inserted into the serpin 2 gene (B13R) locus (Bennett et al., 1999). Interestingly, expression of a firefly luciferase gene inserted in the VACV TK locus of VACV WR was significantly reduced when a functional TK gene from HSV-1 was inserted into the *Hind*III F site (Coupar et al., 2000). One could interpret this result as showing that a functional TK in general may negatively affect the expression of the inserted antigen in WR. However, whether it is due to the insertion into the *Hind*III F site was not formally ruled out by Coupar et al. (2000). Studies relating insertion sites to the immunogenicity of a given antigen are far more limited. In a study by Manuel et al. (2010), the immunogenicity of a human cytomegalovirus antigen expressed by the VACV early/late H5 promoter from two different insertion sites in MVA was examined. In brief, two viruses were compared: one expresses the foreign antigen from the intergenic region between I3L and I4L genes while the other expresses the antigen from the deletion II region. Although both viruses allowed a similar level of foreign antigen expression, the antigen expressed from the I3L/I4L intergenic region induce a more potent CD8$^+$ T cell response than that expressed from the deletion II region (Manuel et al., 2010). This study indicates that an insertion site can affect the immunogenicity of the inserted foreign antigen, independent of the presentation level. Understanding the mechanism responsible might lead to an

identification of better insertion sites for inducing potent $CD8^+$ T cell immunity against foreign antigens.

Our results also suggest that the functional TK expressed from MVA-delIII-SIIN may negatively influence the immunogenicity of the OVA-257 minigene expressed from the delIII region. By deleting the VACV TK from MVA-delIII-SIIN, the OVA-257 minigene induced a similar OVA-257-specific $CD8^+$ T cell response as MVA-delIII-OVA-delTK, the corresponding MVA expressing full-length OVA. VACV TK phosphorylates thymidine to form thymidine monophosphate which is then further processed to create thymidine triphosphate for DNA synthesis (Dubbs and Kit, 1964; Hruby and Ball, 1982; Hruby et al., 1983; Hruby, 1985). This protein is not essential for in vitro VACV replication. However, deletion of TK from WR results in an attenuation of the virus, probably because of the reduced ability for the TK-deleted virus to replicate in non-dividing cells (Buller et al., 1985). A study has demonstrated that a disruption of the TK gene in VACV strain Praha reduced the antibody response elicited against a foreign antigen expressed from the VACV haemagglutinin gene (Kutinová et al., 1999). The decreased antibody response correlated with the reduction of the virulence of the recombinant virus in vivo (Kutinová et al., 1999). In this thesis, the examination of the influence of the TK gene on $CD8^+$ T cell immunity was performed using MVA-based recombinant viruses. It should be noted that MVA does not replicate in vivo and therefore loss of TK would not be predicted to have a change in phenotype in this strain. More importantly, our studies point to a specific effect of TK on the immunogenicity of the inserted minigene constructs, but not that of the native VACV antigens.

One possible explanation of the enhanced immunogenicity of the minigene expressed from a TK-deficient MVA is that deletion of TK may affect VACV gene expression cascade in vivo. Without a functional TK, VACV may not be able to replicate the VACV genome effectively in resting cells in vivo. It has been shown that expression of VACV intermediate and late genes relies on VACV genome replication (Vos and Stunnenberg, 1988; Keck et al., 1990; Baldick and Moss, 1993). Thus, it is possible that the decreased VACV genome replication ability in cells infected with TK-deficient VACV reduces VACV intermediate and

late gene expression and alters the VACV gene expression cascade. This may subsequently lead to an enhanced expression level of transgenes and allow better $CD8^+$ T cell priming. However, this correlation between the gene expression cascade and the immunogenicity of the inserted antigen is not likely. Firstly, as the inserted transgenes were all controlled by the same p7.5 promoter, any changes of the expression level should affect both the minigene and full-length constructs. This should be reflected in the immunogenicity of both constructs, not only that of minigene as observed in our study. Secondly, it has been shown that inhibition of VACV genome replication in vitro with cytosine arabinoside, a DNA replication inhibitor, resulted in decreased expression level of transgenes driven by the VACV p7.5 promoter (Mackett et al., 1984; Cochran et al., 1985). Therefore, any possible changes of expression level resulting from a loss of a functional TK would be detrimental, not beneficial, to the foreign antigens. Thirdly, viral DNA replication does not occur in DCs infected with MVA in vitro (Chahroudi et al., 2006). Therefore, the VACV gene expression cascade may be interrupted in the MVA-infected DCs, independent of the TK expression. Overall, it is unlikely that alteration of VACV gene expression cascade in APCs infected with TK-deficient MVA is responsible for the enhanced immunogenicity of a minigene construct.

An alternative explanation is that the VACV TK protein may have other unknown functions. Many VACV proteins have multiple functions and this could include the VACV TK protein. We have examined whether expression of TK from MVA might affect the survival of the infected cells in vitro (data not shown). Our hypothesis was that if VACV TK had a pro-apoptotic property, removing this protein might extend the survival of the infected APC to allow better direct priming of $CD8^+$ T cell in vivo. However, we failed to detect any obvious changes in the viability or the expression of an apoptotic marker (detection of phosphatidylserine via staining with fluorophore-conjugated Annexin V) between BMDCs infected with MVA-delIII-SIIN and MVA-delIII-SIIN-delTK (data not shown). This suggests that expression of TK does not affect the cell viability during MVA infection. Alternatively, we might have chosen the wrong type of APC for testing this hypothesis. DCs isolated from mice could be used in future studies.

The experiments with the gB-498-expressing VACV showed that the ER-targeted gB-498 minigene reduced $CD8^+$ T cell responses against several native VACV epitopes. This might be due to the competition for MHC-I binding in the infected cells. We have performed an experiment in which DC2.4 cells were co-infected with WR-TK-SIIN together with WR-ESmini-gB, WR-Full-gB or WR-delTK as a control (data not shown). Co-infection of cells with either of the gB-expressing recombinants reduced the OVA-257/MHC-I presentation compared with cells co-infected with WR-TK-SIIN and WR-delTK. However, there was no difference in the OVA-257 presentation level between cells co-infected with WR-ESmini-gB or with WR-Full-gB. This experiment implies that enhanced presentation of gB-498 by the ER-targeted minigene did not significantly influence the MHC-I presentation of peptides from other origins, compared with that processed from the full-length gB antigen. Rather, it is likely that expression of a foreign gene by the VACV p7.5 promoter might suppress the expression of other antigens and thus affect their presentation measured in the in vitro experiment. Another possible reason for the immunodomination effect for the gB-498 minigene is that there is a greater competition between naive $CD8^+$ T cells with different specificities in vivo for their cognate epitopes on APC for priming. A previous study has shown that there are around 500 and 1000 gB-498- and B8-20-specific naive $CD8^+$ T cells in a naive mouse respectively (Haluszczak et al., 2009). These numbers are relative high compared to other naive $CD8^+$ T cell populations that have been examined (Kotturi et al., 2008; Obar et al., 2008; Flesch et al., 2010; La Gruta et al., 2010). Thus, expressing gB-498 as an ER-targeted minigene to facilitate direct presentation may allow more naive gB-498-specific $CD8^+$ T cells to interact with the infected APCs in vivo and they may out-compete the naive $CD8^+$ T cells specific to the native VACV peptides. One way to examine this immunodomination effect is to generate a recombinant virus that co-expresses gB-498 and OVA-257 as two separate minigenes. With this virus, we could study the effect of T cell competition by adoptively transferring different ratios of gB-I and OT-I $CD8^+$ T cells which are specific to gB-498 and OVA-257 peptides respectively (Hogquist et al., 1994; Mueller et al., 2002).

In section 4.2.3, we noticed a decrease of $CD8^+$ T cell priming in general in WR-delB8-miniB8 as determined by the total number of antigen-specific $CD8^+$ T cells

induced. It has been shown that WR-delB8, a WR with B8R gene deleted (Symons et al., 2002), does not show any attenuation in vivo in C57Bl/6 mice. The investigators of this study (Symons et al., 2002) generated a revertant virus based on WR-delB8, by inserting the B8R gene back, to control any possible attenuation caused by secondary unwanted mutations that might be introduced during the generation of the recombinant virus. In a separate study, an independent B8-knockout WR recombinant was generated and it was shown that this virus was less virulent in BALB/c x C57Bl/6 F_1 mice (Verardi et al., 2001). However, no revertant virus was included. For WR-delB8-miniB8 used in this chapter, the reduction of immunogenicity could be due to the introduction of the B8-20 minigene or a random mutation in the viral genome. One approach to discriminate between the two possibilities is to include an independent recombinant in the experiments, similar to the two independent MVA-delIII-SIIN-delTK viruses used in Figure 4-17.

In summary, similar to WR, epitopes expressed as minigenes from MVA to facilitate direct presentation allow effective $CD8^+$ T cell priming in vivo. However, this is dependent on the location of the insertion site of MVA.

Chapter 5 Investigation of mechanisms for the lack of immunogenicity of the influenza A virus NP-366 minigene expressed from vaccinia virus

5.1 Introduction

In Chapter 4 an unexpected finding was observed, the ER-targeted PR8NP-366 minigene was less immunogenic than the full-length PR8NP antigen when expressed from VACV strain WR. This finding not only differs from the results obtained with the other WR recombinants used in Chapter 4, but also contradicts the long-held paradigm that ER-targeted minigenes expressed from WR should be at least as immunogenic, if not more than, full-length constructs (Restifo et al., 1995). Understanding this phenomenon may reveal new aspects of antigen processing pathways.

Full-length NP is the structural protein associated with the segmented single-stranded negative-sense RNA genomes of IAV (Pons et al., 1969; Content and Duesberg, 1970; Compans et al., 1972; Arranz et al., 2012). Multiple NP molecules are bound to individual RNA genome segments to form ribonucleoprotein complexes which are required for viral RNA transcription and replication (together with viral RNA polymerase subunits; Compans et al., 1972; Murti et al., 1988; Coloma et al., 2009; Arranz et al., 2012). Within newly-infected cells, NP is an essential component required for importing ribonucleoprotein complexes into the nucleus, a critical step for viral RNA transcription and replication (Davey et al., 1985; Martin and Helenius, 1991; O'Neill et al., 1995; Neumann et al., 1997; Wang et al., 1997).

There are several immunogenic peptides within IAV NP that can bind to murine or human MHC-I and are recognised by anti-IAV $CD8^+$ T cells during IAV infection (McMichael et al., 1986a; McMichael et al., 1986b; Townsend et al., 1986b; Taylor et al., 1987; Rotzschke et al., 1990; DiBrino et al., 1993; Wu et al., 2011; Grant et al., 2013). One such peptide is NP-366 at position 366-374 and this peptide is restricted to the murine MHC-I $H\text{-}2D^b$ (Rotzschke et al., 1990). This peptide was one of the first mapped peptides found to bind to MHC-I. Multiple natural variants of the NP-366 peptide have been identified in different IAV strains (Zhong et al., 2010). Most research has focused on two variants of this peptide. One is expressed by IAV strain PR8 and it has the amino acid sequence ASNENMETM (Rotzschke et al., 1990; Falk et al., 1991a). In this thesis, the NP

antigen expressed by PR8 is called PR8NP and the antigenic peptide is called PR8NP-366. Another variant of the NP-366 peptide with an amino acid sequence of ASNENMDAM is expressed by the IAV strain NT60 (Townsend et al., 1985; Townsend et al., 1986b). Here, the NP antigen from strain NT60 is named NT60NP and its NP-366 peptide NT60NP-366. It has been shown that PR8NP-366 and NT60NP-366 bind to H-2D^b with similar affinity (Williams et al., 1996; Tourdot et al., 1997). During an acute infection with IAV strain PR8 in C57Bl/6 mice, PR8NP-366 is co-dominant with the IAV PA-224 peptide (Belz et al., 2000). This immunodominance hierarchy changes in the secondary response, in which PR8NP-366 becomes the immunodominant peptide over all other IAV antigenic peptides (Belz et al., 2000; Belz et al., 2001). Similar to PR8NP-366, NT60NP-366 is co-immunodominant with PA-224 in mice immunised with IAV strain NT60 (Cho et al., 2003). An advantage of NT60NP-366 is that this peptide presented on H-2D^b can be recognised by a TCR-transgenic mouse strain, known as the F5 mouse strain (Mamalaki et al., 1992; Mamalaki et al., 1993). Most T cells in F5 transgenic mice express the TCR from a CD8$^+$ T cell clone that is specific for the NT60-NP366/H-2D^b complex (Townsend et al., 1984; Townsend et al., 1985; Townsend et al., 1986b). It should be noted that the F5 CD8$^+$ T cells do not cross react with the PR8NP-366 peptide.

In this chapter, we firstly confirmed and extended our findings from Chapter 4, demonstrating that the CD8$^+$ T cell responses against PR8NP-366 and NT60-NP366 were not effectively primed when they were expressed from VACV strain WR as constructs that allow effective direct presentation. These constructs included a cytosolic minigene, ER-targeted minigene and rapidly-degraded antigen. A series of experiments were then conducted to examine the mechanisms behind these findings.

5.2 Results

5.2.1 The ER-targeted PR8NP-366 minigene expressed from WR allows effective MHC-I presentation of PR8NP-366 on infected cells in vitro

(Viruses used in this chapter are listed in Table A-4 in the Appendix)

Before any further in vivo examination, the direct presentation ability of PR8NP-366 expressed as an ER-targeted minigene from WR was examined. This is an important question because the lack of immunogenicity of the minigene might simply be due to its inability to display the PR8NP-366 peptide on MHC-I. Similar to the PB1F2-62 peptide focused in Section 4.2.2.2, there is a reagent which detects the PR8NP-366/H-2D^b complex on infected cells (Lev et al., 2008). However, it is not sensitive enough to detect the presentation of PR8NP-366 processed from the full-length PR8NP antigen (J.W. Yewdell, personal communication). So, we exploited the in vitro direct presentation assay used in Section 4.2.1.1. In brief, DC2.4 cells were infected with WR-ESmini-PR8NP or WR-Full-PR8NP, which express the ER-targeted PR8NP-366 minigene or the full-length PR8NP antigen respectively. These infected DC2.4 cells were used to stimulate splenocytes isolated from a mouse infected with IAV strain PR8 seven days earlier. There are peptides other than PR8NP-366 in the full-length PR8NP antigen that may be presented on the WR-Full-PR8-NP-infected DC2.4 cells, but it has been shown that these peptides are not immunogenic and cannot be recognised by the CD8$^+$ T cells isolated from mice infected with IAV strain PR8 (Zhong et al., 2003). In addition, similar to the result shown in Figure 4-5, the WR-delTK-infected cells did not re-stimulate the IAV-immune CD8$^+$ T cells. This confirms that there was no cross-reactivity between the IAV-specific CD8$^+$ T cells and the native VACV peptides presented on the infected DC2.4 cells (Figure 5-1).

As seen in Figure 5-1, DC2.4 cells infected with WR-ESmini-PR8NP re-stimulated more CD8$^+$ T cells isolated from the IAV-infected mouse to produce IFN-γ than cells infected with WR-Full-PR8NP when stimulator:effector ratios of 1:5, 1:25 or 1:50 were used. A similar level of stimulation was detected when a stimulator:effector ratio of 1:1 was used. This data suggested that PR8NP-366 expressed as an ER-targeted minigene allowed at least as much, if not more, direct presentation of PR8NP-366 on the surface of infected cells as the full-length

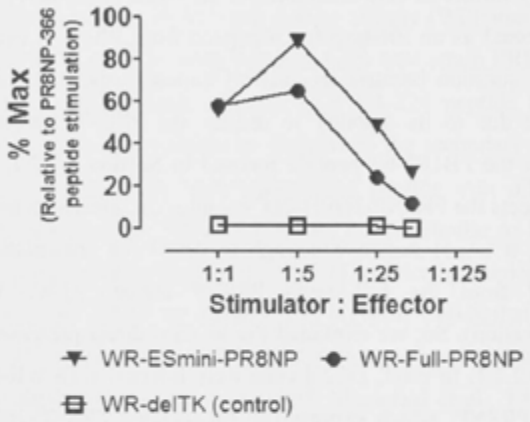


Figure 5-1| Expressing PR8NP-366 as an ER-targeted minigene from VACV WR allows effective direct presentation. DC2.4 cells were infected with the indicated recombinant WR at a m.o.i. of 5 for 5 h. The infected cells (stimulators) were then co-cultured with splenocytes (effectors) from a mouse immunised i.p. with 500 hemagglutinin units (HAU) of IAV strain PR8 seven days before, at different stimulator:effector ratios. BFA was added 1 h later, followed by another 3 h of co-culturing. ICS was then performed to measure the percentage of $CD8^+$ T cells that produced IFN-γ. A standard ICS assay with a synthetic PR8NP-366 peptide was also performed in parallel to measure the total PR8NP-366-specific T cell response. Data show the level of PR8NP-366-specific $CD8^+$ T cell activation by the infected DC2.4 cells, after being standardised to the percentage of maximal activation as induced by the synthetic PR8NP-366 peptide. Data are representative of three independent experiments. Similar results were also obtained when 293-D^b cells or BMDCs were used as stimulators.

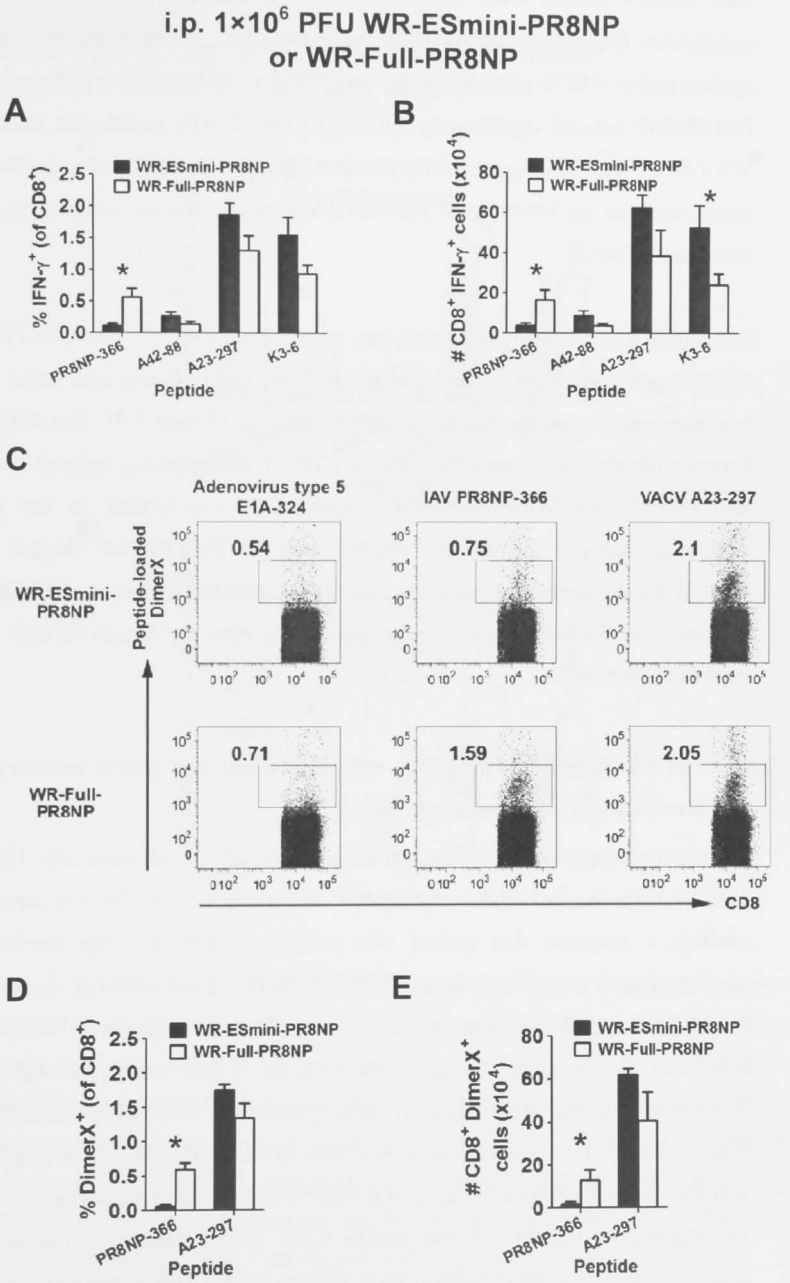
PR8NP antigen. As well as DC2.4 cells, BMDCs and 293-D^b, a human 293A cell line stably expressing H-$2D^b$ (Tscharke et al., 2005), have been used as stimulators in this assay. Experiments with these cells also produced similar results as those shown in Figure 5-1 (data not shown). Overall, this experiment indicates that PR8NP-366 is not defective in direct presentation when expressed as an ER-targeted minigene, compared to the full-length PR8NP antigen.

5.2.2 PR8NP-366 expressed as a minigene from WR is less able to prime $CD8^+$ T cells than full-length PR8NP

The data shown in Figure 4-4D represented only one single experiment. To confirm this result, the experiment was repeated here by measuring anti-PR8NP-366 $CD8^+$ T cell responses in mice immunised with WR-ESmini-PR8NP or WR-Full-PR8NP seven days post immunisation. In addition to the standard ICS assay, DimerX loaded with the PR8NP-366 peptide was used to enumerate the PR8NP-366-specific $CD8^+$ T cells. Similar to peptide/MHC-I tetramers (Altman et al., 1996), peptide-loaded DimerX binds to TCRs that recognise the loaded peptide (Flesch et al., 2012). Importantly, this assay does not rely on the ability of $CD8^+$ T cells to respond to ex vivo peptide stimulation, allowing us to detect a peptide-specific $CD8^+$ T cell response independent of the functionality of the cells.

Seven days after immunisation, WR-ESmini-PR8NP elicited a PR8NP-366-specific response of around 0.15% of splenic $CD8^+$ T cells as determined by ex vivo peptide stimulation and ICS (Figure 5-2A). This was significantly lower than the response induced by WR-Full-PR8NP, in which around 0.55% of splenic $CD8^+$ T cells were specific to PR8NP-366. A similar result was observed when the total number of responding $CD8^+$ T cells was calculated (Figure 5-2B). In addition, the data obtained from PR8NP-366-loaded DimerX staining confirmed the ICS results, in which a much smaller anti-PR8NP-366 $CD8^+$ T cell population was induced by the ER-targeted minigene, compared to the full-length antigen (Figure 5-2C, D and E). It should be noted that results shown in Figure 5-2D and E were calculated by subtracting the background values determined by using DimerX loaded with an irrelevant adenovirus type 5 E1A-324 peptide (sequence SGPSNTPPEI; Kast and Melief, 1991) from the measured values determined

Figure 5-2| The full-length IAV PR8NP antigen is more immunogenic than the ER-targeted PR8NP-366 minigene for inducing a PR8NP-366-specfic $CD8^+$ T cell response in vivo. Groups of three mice were immunised i.p. with 1×10^6 PFU of WR-ES-mini-PR8NP or WR-Full-PR8NP. Seven days later, splenocytes from the immunised mice were either stimulated with peptides ex vivo followed by ICS (A and B) or stained with DimerX loaded with the indicated peptides (C, D and E). Percentages (A) and absolute numbers (B) of IFN-γ-producing cells after stimulation with peptides indicated are shown. (C) Representative flow cytometric plots showing $CD8^+$ T cells from mice immunised with WR-ESmini-PR8NP (first row) or WR-Full-PR8NP (second row) labelled with DimerX loaded with adenovirus type 5 E1A-324 (first column), IAV PR8NP-366 (second column) or VACV A23-297 peptides (third column). The number on each plot shows the percentage of $DimerX^+$ cells of the $CD8^+$ T cell population. (D and E) Percentages (D) and absolute numbers (E) of $CD8^+$ T cells stained with DimerX loaded with peptides indicated were shown. Backgrounds were determined by using DimerX loaded with adenovirus type 5 E1A-324 peptide and these values were subtracted from the measured values using DimerX loaded with the peptides indicated. (A, B, D and E) Data represent results compiled from two independent experiments (mean ± SEM; n = 6). * denotes statistical significance ($p < 0.05$).


i.p. 1×10^6 PFU WR-ESmini-PR8NP
or WR-Full-PR8NP
A
B
WR-ESmini-PR8NP
WR-Full-PR8NP
% IFN-γ+ (of CD8+)
# CD8+ IFN-γ+ cells (x10^4)
PR8NP-366
A42-88
A23-297
K3-6
Peptide
C
Adenovirus type 5 E1A-324
IAV PR8NP-366
VACV A23-297
Peptide-loaded DimerX
0.54
0.75
2.1
0.71
1.59
2.05
CD8
D
E
% DimerX+ (of CD8+)
# CD8+ DimerX+ cells (x10^4)

with DimerX loaded with VACV A23-297 or PR8NP-366 peptides. In this experiment, there were variations between the $CD8^+$ T cell responses induced against native VACV peptides by the two WR recombinants. For instance, WR-Full-PR8NP induced significantly fewer K3 $CD8^+$ T cells specific for K3-6 than WR-ESmini-PR8NP (Figure 5-2B). However, this variation should not affect our conclusion that the ER-targeted PR8NP-366 minigene is less immunogenic than full-length PR8NP.

In addition to the ER-targeted minigene, we also examined the cytosolic PR8NP-366 minigene expressed by WR-mini-PR8NP and this minigene also had a lower immunogenicity than the full-length PR8NP antigen (Figure 5-3). The difference between the size of the anti-PR8NP-366 $CD8^+$ T cell response induced by WR-mini-PR8NP and WR-Full-PR8NP (Figure 5-3) was similar to the 4-fold difference between WR-ESmini-PR8NP and WR-Full-PR8NP (Figure 5-2). Overall, these experiments confirm that the ER-targeted and cytosolic minigene constructs of PR8NP-366 are less immunogenic than full-length PR8NP when expressed from WR.

5.2.3 The ER-targeted PR8NP-366 minigene elicits less potent memory and recall $CD8^+$ T cell responses

The previous experiments focused on the primary $CD8^+$ T cell responses. Here we examined whether the ER-targeted PR8NP-366 minigene was also less capable of eliciting a response that persist into memory. Seven to eight weeks post immunisation, a much smaller anti-PR8NP-366 $CD8^+$ T cell response was induced by the ER-targeted minigene than the full-length antigen. By using the standard ICS assay, we detected around 0.05% of $CD8^+$ T cells that responded to the PR8NP-366 peptide stimulation in mice immunised with WR-ESmini-PR8NP (Figure 5-4A). In comparison, around 0.18% of the total $CD8^+$ T cell population was specific to PR8NP-3366 after WR-Full-PR8NP immunisation (Figure 5-4A). The response of the PR8NP-366-specific $CD8^+$ T cells measured as an absolute number produced a similar observation (Figure 5-4B). This experiment suggests that in addition to the reduced acute response, the ER-targeted minigene also induces a smaller memory response.

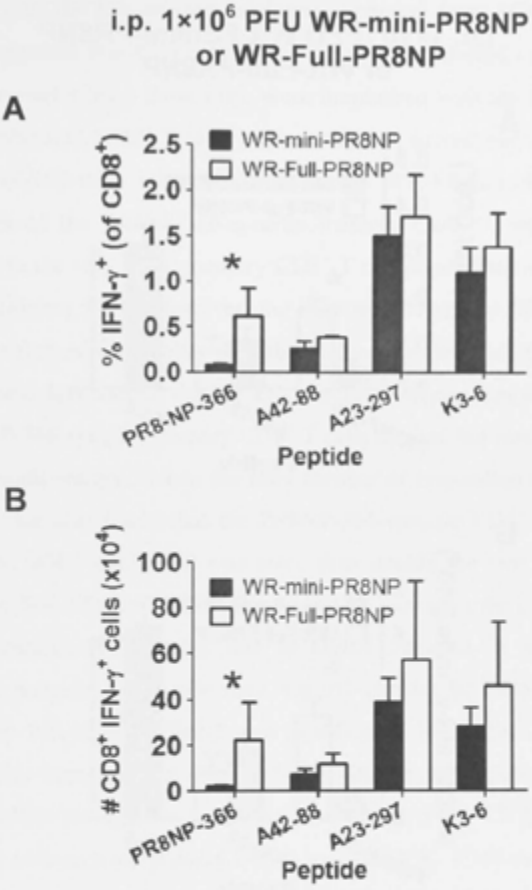


Figure 5-3| The cytosolic PR8NP-366 minigene induces a significantly lower PR8NP-366-specific CD8$^+$ T cell response than the full-length PR8NP antigen when expressed from WR. Mice were immunised i.p. with 1×10^6 PFU of WR-mini-PR8NP or WR-Full-PR8NP. Seven days later, antigen-specific CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A) and absolute numbers (B) of CD8$^+$ T cells responding to the indicated peptides are shown as mean ± SEM. Results are compiled from two independent experiments (WR-mini-PR8NP: n = 6; WR-Full-PR8NP: n = 2). * denotes statistical significance ($p < 0.05$).

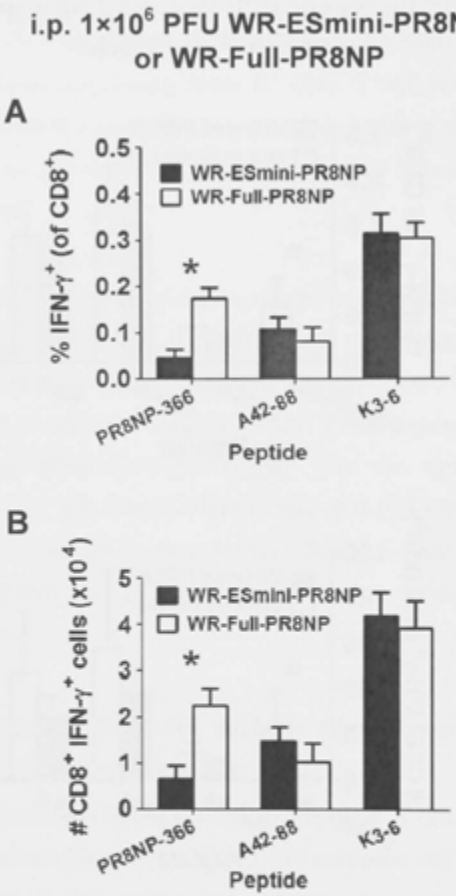


Figure 5-4| PR8NP-366 expressed as an ER-targeted minigene is less able to induce a memory CD8$^+$ T cell response than its full-length counterpart. Groups of three mice were immunised i.p. with 1×10^6 PFU of WR-ES-mini-PR8NP or WR-Full-PR8NP. After 7 to 8 weeks, splenocytes from the immunised mice were isolated and stimulated with peptides ex vivo followed by ICS. Data are shown as percentages (A) or absolute numbers (B) of CD8$^+$ T cells responding to the peptides indicated and are compiled from two independent experiments (as mean ± SEM ; n = 6). * denotes statistical significance ($p < 0.05$).

The ability of the ER-targeted minigene expressed from WR to re-stimulate a memory response was then determined. Mice were infected i.p. with IAV strain PR8. Four weeks later, these mice were immunised with the PR8NP-expressing WR recombinants. $CD8^+$ T cell responses against several native VACV peptides and PR8NP-366 were measured 7 days later. WR-ESmini-PR8NP allowed re-stimulation of the PR8NP-366-specific memory $CD8^+$ T cells in vivo, when compared to the very small memory $CD8^+$ T cell population induced by the IAV infection (Figure 5-5A). However, the response induced by WR-ESmini-PR8NP remained substantially smaller than that induced by WR-Full-PR8NP, by around 2-fold. These data showed that the ER-targeted minigene was able to re-stimulate the PR8NP-366-specific memory $CD8^+$ T cells in vivo, but was less efficient than the full-length antigen. When the total number of responding $CD8^+$ T cells was measured, we also found that the PR8NP-366-specific $CD8^+$ T cell population induced by WR-Full-PR8NP was more than double the size of the population induced by WR-ESmini-PR8NP (Figure 5-5B). However, the difference observed was not significant, probably due to higher variation in responses amongst individual animals. As a side note, the B8-20 specific $CD8^+$ T cell response induced by WR-ESmini-PR8NP was significantly higher than that of WR-Full-PR8NP after immunisation (Figure 5-5A). One possible explanation is that the strong PR8NP-366-specific recall response induced by WR-Full-PR8NP might negatively influence the primary B8-20-specific $CD8^+$ T cell priming. It has been previously shown that prior priming with IAV does not influence the immunogenicity of native VACV peptides induced by immunisation with WR expressing an ER-targeted IAV PA-224 minigene (WR-ESmini-PA; Wang et al., 2009). Therefore, this may be an antigen-specific phenomenon. It is likely that the size of the response against the IAV peptide examined plays a role here as it seems that the PR8NP-366-specific response re-stimulated by WR-Full-PR8NP (as shown in Figure 5-5) was bigger than the response against PA-244 re-stimulated by WR-ESmini-PA (as shown in Wang et al. 2009).

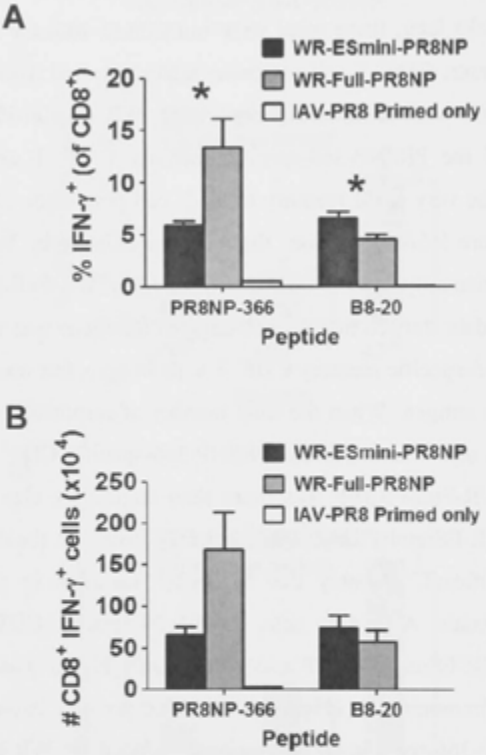


Figure 5-5| ER-targeted PR8NP-366 minigene elicits a smaller secondary $CD8^+$ response than the full-length PR8NP antigen. Mice were infected with 500 HAU IAV-PR8 via the i.p. route. Four weeks later, these mice were either left un-immunised or immunised with 1×10^6 PFU of the indicated recombinant WR. Seven days after the VACV immunisation, epitope-specific $CD8^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Results are shown as percentage (A) or absolute number (B) of $CD8^+$ T cells specific to the indicated peptides. Data are compiled from two independent experiments (mean ± SEM; WR-ESmini-PR8NP and WR-Full-PR8NP: n = 5; IAV-PR8 primed only: n = 2). * denotes statistical significance ($p < 0.05$).

5.2.4 The ER-targeted NT60NP-366 minigene elicits a smaller NT60NP-366-specific CD8$^+$ T cell response in vivo

To investigate whether the observations above were specific to PR8NP-366 or if they also applied to other NP-366 variants, the immunogenicity of NT60NP-366 expressed as a minigene from WR was examined. In addition, NT60NP-366 presented on H-2D^b can be recognised by F5 TCR transgenic CD8$^+$ T cells as discussed in Section 5.1 (Mamalaki et al., 1992; Mamalaki et al., 1993). With this tool, events that occurred early after priming could be traced. Two WR recombinants were used, namely WR-ESmini-NT60NP and WR-Full-NT60NP. The former recombinant virus expresses NT60NP-366 as an ER-targeted minigene. It should be noted that the ER-targeting sequence is derived from the signal sequence of the IAV hemagglutinin protein (Elliott et al., 1995). WR-Full-NT60NP expresses the full-length NT60 NP antigen.

The direct presentation capacity of the ER-targeted NT60NP-366 minigene expressed from WR was first measured with an in vitro direct presentation assay similar to that shown in Figure 5-1 with some modifications. Instead of using splenocytes from infected mice as effectors, in vitro-activated F5 CD8$^+$ T cells were used. To generate these activated cells, splenocytes from a F5 mouse were stimulated with NT60NP-366 peptide in the presence of IL-2 for 4 days. The in vitro-activated CD8$^+$ T cells were mixed with C57Bl/6 BMDCs that were infected with WR-ESmini-NT60NP, WR-Full-NT60NP or WR-delTK. The level of re-stimulation of the activated F5 CD8$^+$ T cells was detected as the frequency of IFN-γ-producing CD8$^+$ T cells using ICS and the result is shown in Figure 5-6. BMDCs infected with the control virus, WR-delTK, did not restimulate the F5 CD8$^+$ T cells, demonstrating that these TCR-transgenic cells did not cross-react with the presented native VACV peptides. Compared to WR-Full-NT60NP, BMDCs infected with WR-ESmini-NT60NP re-stimulated a larger population of activated F5 CD8$^+$ T cells (Figure 5-6). This suggests that the ER-targeted NT60NP-366 minigene expressed from WR allows better direct presentation of the NT60NP-366 peptide on infected cells than the full-length NT60NP antigen.

It should be noted that the in vitro activation of F5 cells with the cognate NT60NP-366 peptide is not very efficient. After in vitro activation, only around

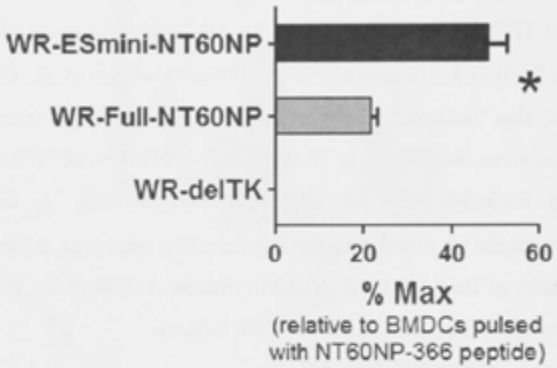


Figure 5-6| The in vitro direct presentation efficiency of NT60NP-366 is improved when it is expressed as an ER-targeted minigene from WR, compared to the full-length NT60NP antigen. Splenocytes from a TCR-transgenic F5 mouse were isolated and cultured with the NT60NP-366 peptide in the presence of IL-2 for three days to activate F5 $CD8^+$ T cells. In-vitro-cultured BMDCs from a C57Bl/6 mouse were infected with the indicated recombinant WR at a m.o.i. of 5 for 5 h. The infected BMDCs (stimulators) were then co-cultured with the activated F5 cells (effectors) at a stimulator:effector ratio of 1:1. BFA was added 1 h after cells were mixed, followed by another 3 h of co-culturing. ICS was then performed to measure the percentage of F5 $CD8^+$ T cells that produced IFN-γ. The maximum level of responding F5 $CD8^+$ T cells was determined by simulation with BMDCs pulsed with 1μM of the NT60NP-366 peptide. Data show the level of IFN-γ-producing F5 $CD8^+$ T cells after stimulation with the infected BMDCs, normalised to the percentage of maximal potential stimulation as induced by the NT60NP-366-pulsed BMDCs. Data for the NT60NP-366-expressing recombinants are shown as mean ± SEM from triplicate samples while data for WR-delTK (control) represents a single measurement. Data are representative of two independent experiments. * denotes statistical significance ($p < 0.05$).

15% of F5 $CD8^+$ T cells could respond to stimulation with BMDCs pulsed with the synthetic NT60NP-366 peptide (data not shown). For comparison, this protocol has also been used to activate $CD8^+$ T cells from OT-I TCR transgenic mice, which are specific to OVA-257/H-$2K^b$ (Hogquist et al., 1994). At least 50% of the in vitro-activated OT-I $CD8^+$ T cells would produce IFN-γ after a short period of re-stimulation with the synthetic OVA-257 peptide as measured by ICS (data not shown). The reduced ability of the F5 $CD8^+$ T cells to be activated in vitro as measured in our experiments was in agreement with a previous study which suggested that in vitro activation of F5 $CD8^+$ T cells requires additional inflammatory signals (Schell et al., 2010).

The in vivo immunogenicity of WR-Full-NT60NP and WR-ESmini-NT60NP was then checked. Seven days after immunisation, mice immunised with WR-ESmini-NT60NP had a significantly smaller population of $CD8^+$ T cells specific for NT60NP-366 than those immunised with WR-Full-NT60NP (Figure 5-7). The full-length NT60NP antigen elicited an anti-NT60NP-366 response of around 0.2% of $CD8^+$ T cells in spleens (equivalent to around 6×10^4 NT60NP-366-specific $CD8^+$ T cells), which was around 6-fold higher than that the response induced by the ER-targeted minigene. This suggests that the phenomenon of the low immunogenicity of a minigene is not specific to the PR8NP-366 petpide, but also applicable to the NT60NP-366 peptide.

5.2.5 The ER-targeted NT60NP-366 minigene elicits a smaller F5 TCR transgenic $CD8^+$ T cell response in recipient mice

We then investigated whether F5 $CD8^+$ T cells would behave like the endogenous polyclonal $CD8^+$ T cells in mice immunised with WR-ESmini-NT60NP. $CD8^+$ T cells were purified from F5 mice using negative magnetic bead separation. As the F5 mice were on a C57Bl/6 background, they carry the CD45.2 allelic marker. 5×10^3 isolated F5 $CD8^+$ T cells were adoptively transferred via i.v. injection into B6.SJL mice, which carry the CD45.1 congenic marker. Mice were immunised with WR-Full-NT60NP, WR-ESmini-NT60NP or WR delTK one day later. Seven days post-immunisation, endogenous and transferred F5 $CD8^+$ T cell responses were measured. We found that WR-ESmini-NT60NP induced a smaller

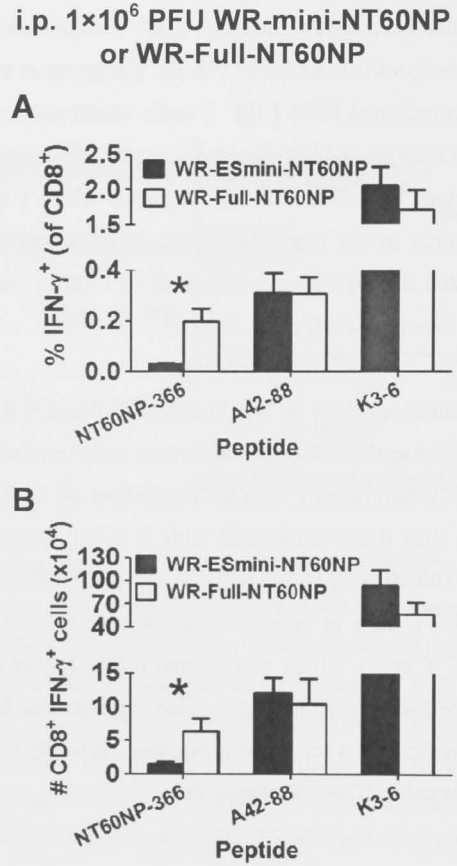


Figure 5-7| Similar to the PR8NP-366 epitope, NT60NP-366 expressed as an ER-targeted minigene elicits a smaller NT60NP-366-specific CD8$^+$ T cell response than the full-length NT60NP antigen when expressed from WR. Groups of three mice were immunised i.p. with the indicated WR recombinants (at a dose of 1×10^6 PFU/mouse). Seven days after the immunisation, CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Results are shown as percentages (A) or absolute numbers (B) of CD8$^+$ T cells specific to the peptides indicated. Data represent results compiled from two independent experiments (mean ± SEM; n = 6). * denotes statistical significance ($p < 0.05$).

population of IFN-γ-producing F5 $CD8^+$ T cells than WR-Full-NT60NP after ex vivo peptide stimulation (F5 cells were $CD45.2^+$; Figure 5-8A and B). The difference was significant when the response was measured either as the percentage of IFN-γ^+ F5 $CD8^+$ T cells in relation to the overall $CD8^+$ T cell population (Figure 5-8A) or as the total number of responding F5 $CD8^+$ T cells (Figure 5-8B). In addition to the reduction of the transferred F5 $CD8^+$ T cell response, the endogenous anti-NT60NP-366 $CD8^+$ T cell response ($CD45.2^-$) was also significantly lower in mice immunised with WR-ESmini-NT60NP, compared with mice immunised with WR-Full-NT60NP (Figure 5-8C and D). This demonstrates that the F5 $CD8^+$ T cells accurately reflects the phenomenon observed in the endogenous response.

The kinetics of the transferred F5 $CD8^+$ T cell responses in the recipient mice were also measured. Similar percentages (0.2-0.3%) and numbers ($2\text{-}4\times10^4$) of IFN-γ-producing F5 $CD8^+$ T cells were detected in mice immunised with WR-ESmini-NT60NP or WR-Full-NT60NP at an early time point, four days post immunisation (Figure 5-9A and B). For WR-Full-NT60NP, the F5 $CD8^+$ T cell response peaked at day 6 after immunisation at approximately 2.2% of total $CD8^+$ T cells and then reduced to around 1% eight days after immunisation (Figure 5-9A). In contrast to WR-Full-NT60NP, the percentage of IFN-γ-producing F5 $CD8^+$ T cells relative to the total $CD8^+$ T cell population in mice immunised with WR-ESmini-NT60NP plateaued from 6 days after immunisation, remaining at around 0.3%. Compared to WR-Full-NT60NP, the transferred F5 responses, as measured as percentage of IFN-γ-producing F5 $CD8^+$ T cells of the total $CD8^+$ T cells, at six and eight days post-immunisation were significantly lower in mice immunised with WR-ESmini-NT60NP (Figure 5-9A). A similar situation was seen when the total number of IFN-γ-producing F5 $CD8^+$ T cells was measured (Figure 5-9B).

An interesting finding about the ability of the transferred F5 $CD8^+$ T cells to produce IFN-γ was also noticed. After stimulation with NT60NP-366 peptide ex vivo, more than 70% of the $CD45.2^+$ F5 $CD8^+$ T cells from WR-Full-NT60NP-immunised mice produced IFN-γ (Figure 5-9C). In contrast, in mice immunised with WR-ESmini-NT60NP, a significantly lower percent of F5 $CD8^+$ T cells

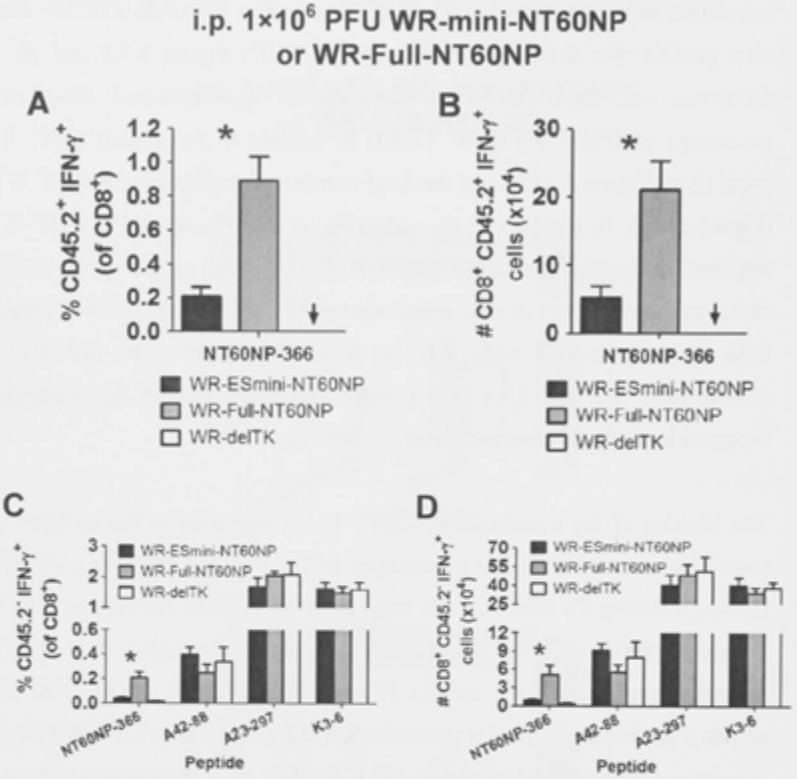


Figure 5-8| The NT60NP-366 minigene expressed from WR primes a smaller F5 TCR-transgenic CD8$^+$ T cell response than the full-length NT60NP antigen. CD8$^+$ T cells from a naive F5 TCR-transgenic mouse, which express the CD45.2 allelic marker, were isolated using magnetic bead separation. 5×10^3 isolated F5 CD8$^+$ T cells were i.v. injected into B6.SJL mice (CD45.1$^+$). One day later, these mice were immunised i.p. with the indicated recombinant WR at a dose of 1×10^6 PFU. Seven days after the immunisation, splenocytes from the immunised mice were stimulated ex vivo with the synthetic peptides as indicated. These cells were labelled with FITC-conjugated anti-CD45.2 and PE-conjugated anti-CD8 antibodies, followed by standard ICS. The transferred CD8$^+$ CD45.2$^+$ cells producing IFN-γ after ex vivo stimulation with the NT60NP-366 peptide are shown as a percentage of the total CD8$^+$ population (A) or as an absolute number (B). Endogenous CD45.2$^-$ CD8$^+$ T cell responses specific to peptides indicated are shown as percentages of total CD8$^+$ T cells (C) or as an absolute numbers (D). Data represent results compiled from two independent experiments (mean ± SEM; WR-ESmini-NT60NP and WR-Full-NT60NP: n = 6; WR-delTK: n = 2). * denotes statistical significance ($p < 0.05$).

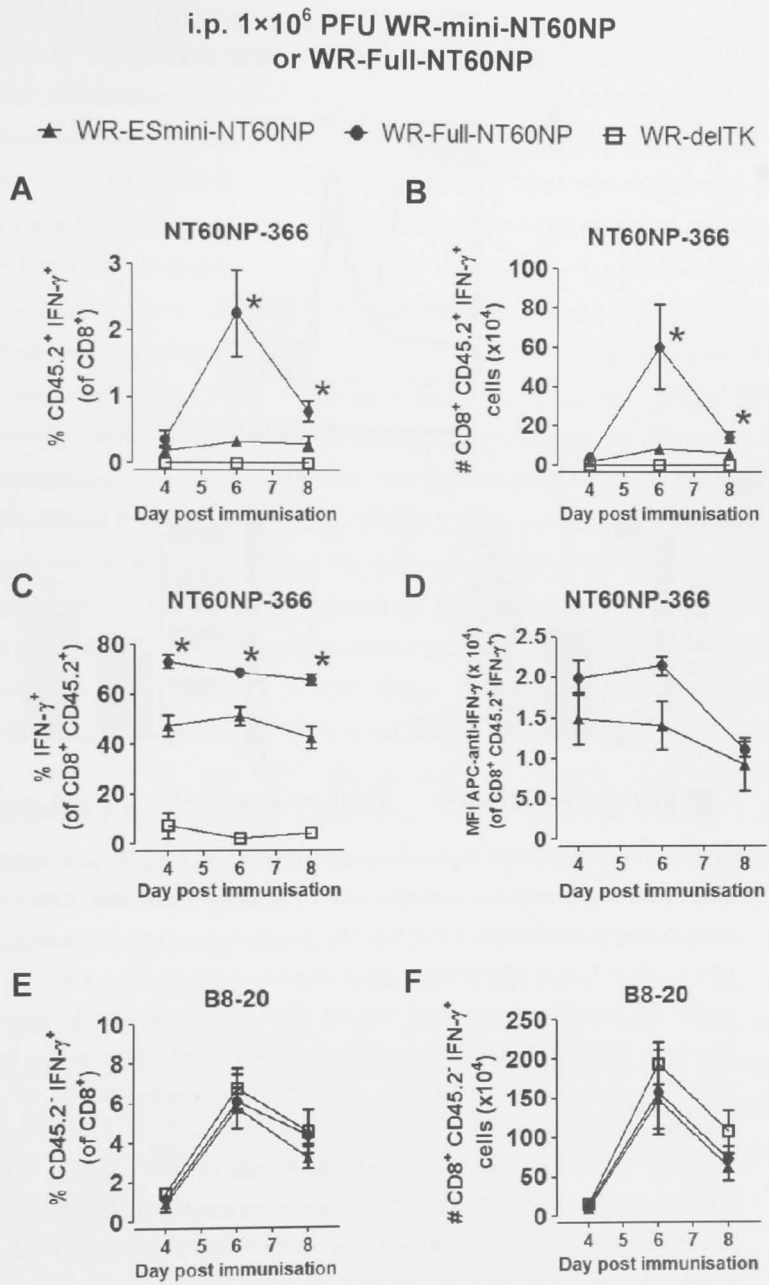
(around 40-50%) could produce IFN-γ after cognate peptide stimulation. Although it is not statistically significantly, $CD8^+$ T cells induced by WR-ESmini-NT60NP seemed to express lower level of IFN-γ than those primed by WR-Full-NT60NP (as measured by MFI; Figure 5-9D). This implies that the F5 $CD8^+$ T cells induced by WR-ESmini-NP60NP may not be as functional. To control for the level of infection across the viruses used, the endogenous ($CD45.2^-$) B8-20-specific $CD8^+$ T cell responses were measured. The responses elicited by the three WR recombinants were similar over the course of immunisation (Figure 5-9E and F). Overall, the data here suggests that the ER-targeted NT60NP-366 minigene induces a smaller and less responsive F5 $CD8^+$ T cell response.

5.2.6 The ER-targeted NT60NP-366 minigene allows a similar level of early activation and proliferation of F5 $CD8^+$ T cells as the full-length NT60NP

We tracked whether the reduction of F5 $CD8^+$ T cell response induced by the ER-targeted NT60NP minigene was caused by a defective activation early during immunisation. One million purified F5 $CD8^+$ T cells were injected i.v. into B6.SJL mice, followed by immunisation with WR recombinants one day later. Splenocytes were isolated one day after immunisation and the expression level of the early activation marker CD69 on the transferred F5 $CD8^+$ T cells was determined (Figure 5-10A). The results shown in Figure 5-10B illustrate that the percentage of F5 $CD8^+$ T cells expressing CD69 in mice immunised with WR-ESmini-NT60NP seemed to be lower, but it was not significantly different from those immunised with WR-Full-NT60NP. Similarly, there was no statistical difference between the expression level of CD69 on the F5 $CD8^+$ cells that were $CD69^+$ (as measured by MFI; Figure 5-10C). Because F5 $CD8^+$ T cells in mice immunised with WR-delTK did not up-regulate CD69, the activation by NT60NP-366-expressing viruses was peptide-specific.

The magnitude of a $CD8^+$ T cell response induced depends on the proliferation of responding $CD8^+$ T cells during the priming phase. Furthermore, CD25, the IL-2 receptor α-subunit whose expression is induced early during $CD8^+$ T cell activation (Kalia et al., 2010), is required for optimal priming of primary anti-

Figure 5-9| A smaller and less reactive F5 transgenic CD8$^+$ T cell response is elicited by the NT60NP-366 minigene when expressed from WR. Isolated 5×10^3 CD45.2$^+$ F5 CD8$^+$ T cells were adoptively transferred via i.v. injection into CD45.1$^+$ B6.SJL mice. One day later, these mice were immunised i.p. with the indicated WR recombinants at a dose of at a dose of 1×10^6 PFU. At times indicated, splenocytes from the immunised mice were stimulated ex vivo with synthetic NT60NP-366 or B8-20 peptides. Cells were then labelled with FITC-conjugated anti-CD45.2 and PE-conjugated anti-CD8 antibodies, followed by standard ICS. Responses were determined as percentages of CD8$^+$ CD45.2$^+$ cells producing IFN-γ relative to the overall CD8$^+$ population (A) or as absolute numbers (B). (C) Percentages of CD8$^+$ CD45.2$^+$ F5 T cells that produced IFN-γ after ex vivo stimulation with the NT60NP-366 peptide. (D) MFI of APC-anti-IFN-γ of the CD8$^+$ CD45.2$^+$ IFN-γ$^+$ cells after ex vivo stimulation with the NT60NP-366 peptide. (E and F) Endogenous CD45.2$^-$ CD8$^+$ T cell responses specific to B8-20 was also determined and are shown as percentages of total CD8$^+$ T cells (E) or as absolute numbers (F). (A-C, E-F) Data represent results compiled from two independent experiments (mean ± SEM; WR-ESmini-NT60NP and WR-Full-NT60NP: n = 6; WR-delTK: n = 2). Data are representatives from two independent experiments. * denotes statistical significance ($p < 0.05$).


i.p. 1×10^6 PFU WR-mini-NT60NP
or WR-Full-NT60NP
WR-ESmini-NT60NP
WR-Full-NT60NP
WR-delTK
A
NT60NP-366
% CD45.2+ IFN-γ+ (of CD8+)
Day post immunisation
B
NT60NP-366
CD8+ CD45.2+ IFN-γ+ cells (x10^4)
Day post immunisation
C
NT60NP-366
% IFN-γ+ (of CD8+ CD45.2+)
Day post immunisation
D
NT60NP-366
MFI APC-anti-IFN-γ (x 10^4) (of CD8+ CD45.2+ IFN-γ+)
Day post immunisation
E
B8-20
% CD45.2- IFN-γ+ (of CD8+)
Day post immunisation
F
B8-20
CD8+ CD45.2- IFN-γ+ cells (x10^4)
Day post immunisation

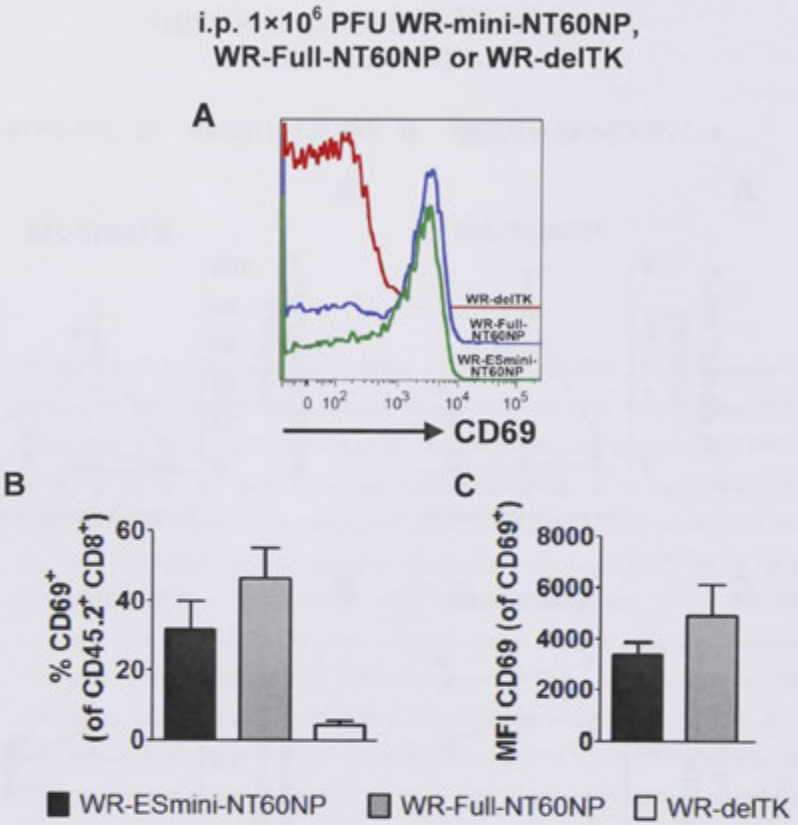
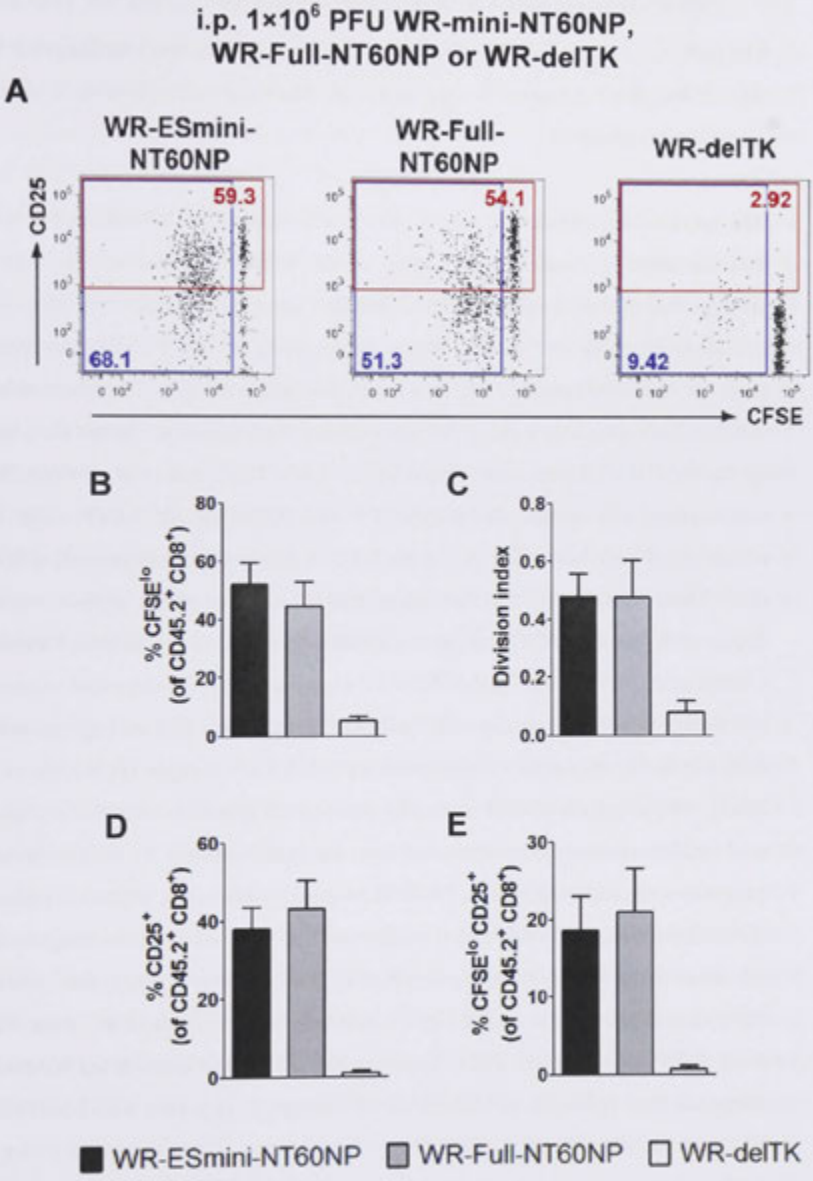


Figure 5-10| NT60NP-366 expressed as an ER-targeted minigene or a full-length antigen from WR activates a similar level of NT60NP-366-specific CD8$^+$ T cells early during immunisation. 1×10^6 F5 CD8$^+$ T cells were adoptively transferred into CD45.1$^+$ B6.SJL mice. One day later, these mice were immunised i.p. with 1×10^6 PFU of the WR recombinants indicated. One day later, splenocytes from the immunised mice were labelled with anti-CD45.2, anti-CD8 and anti-CD69 antibodies. The labelled cells were then analysed with flow cytometry. (A) A representative histogram illustrating the CD69 expression level of the gated CD8$^+$ CD45.2$^+$ T cells from three individual mice, each immunised with WR-ESmini-NT60NP (green), WR-Full-NT60NP (blue), or WR-delTK (red). Percentages of the transferred CD8$^+$ CD45.2$^+$ cells that were CD69$^+$ are shown in (B) while the MFI of CD69 within the CD45.2$^+$ CD8$^+$ CD69$^+$ cells are shown in (C). The results in (B and C) are shown as mean ± SEM and represent compiled data from four independent experiments (WR-ESmini-NT60NP and WR-Full-NT60NP: n = 12; WR-delTK: n = 4).

VACV $CD8^+$ T cell responses (Obar et al., 2010). Therefore, the ability of the ER-targeted NT60NP-366 minigene and the full-length NT60NP antigen to drive early proliferation and CD25 expression by F5 $CD8^+$ T cells in vivo was investigated. B6.SJL mice were adoptively transferred with 1×10^6 F5 $CD8^+$ T cells that were labelled with CFSE and the mice were immunised with WR recombinants one day later. Two days after immunisation, splenocytes were collected and the proliferation of and CD25 expression by F5 $CD8^+$ T cells was determined by flow cytometric analysis. After a round of cell division, a CFSE-labelled cell gives rise to two daughter cells with half the initial amount of CFSE (Lyons and Parish, 1994). Therefore, F5 $CD8^+$ T cells with reduced CFSE intensity represent cells that have undergone proliferation. In this assay, the percentage of $CFSE^{lo}$ F5 $CD8^+$ T cells was similar in mice immunised with either WR-ESmini-NT60NP or WR-Full-NT60NP (Figure 5-11A and B). In addition, there was no difference between the extent of division of the F5 $CD8^+$ T cells induced by the ER-targeted minigene or the full-length antigen. This was shown by the similar division index, which represents the average number of division for every transferred F5 $CD8^+$ T cell (Figure 5-11C). Moreover, WR-ESmini-NT60NP and WR-Full-NT60NP induced a similar percentage of F5 $CD8^+$ T cells to express CD25 two days after immunisation (Figure 5-11A and D). Furthermore, the percentages of $CFSE^{lo}$ $CD25^+$ cells within the F5 $CD8^+$ T cell population were similar between mice immunised with WR-ESmini-NT60NP and WR-Full-NT60NP (Figure 5-11E). Collectively, results in Figures 5-10 and 5-11 demonstrates that early activation and proliferation of NT60NP-366-specific $CD8^+$ T cells induced by the ER-targeted NT60NP-366 minigene are not significantly different from that of the full-length NT60NP antigen. These data suggests that any differences happening early after immunisation must be subtle. In addition, our data points to a role of the differences in proliferation and/or survival of F5 $CD8^+$ T cells during the later stage of immunisation in affecting the outcome observed.

Figure 5-11| NT60NP-366 expressed as an ER-targeted minigene or as a full-length antigen from WR allows a similar level of proliferation and activation of F5 TCR transgenic CD8$^+$ T cells early after immunisation. CFSE-labelled CD45.2$^+$ F5 CD8$^+$ T cells were adoptively transferred into CD45.1$^+$ B6.SJL mice (1×10^6 cells per mouse). One day later, mice were immunised i.p. with at 1×10^6 PFU of the viruses indicated. Two days post immunisation, expression of CD25 and the CFSE level of the transferred F5 CD8$^+$ T cells from the immunised mice were analysed by flow cytometry. (A) Representative flow cytometric plots illustrating the CFSE and CD25 levels of the transferred CD45.2$^+$ CD8$^+$ F5 cells from mice immunised with viruses indicated. On each plot, CD25$^+$ population is gated with a red-coloured gate, and the number in red represents the percentage of CD25$^+$ events of the CD8$^+$ CD45.2$^+$ population. The CFSE^{lo} population is gated with a blue-coloured gate, and the number in blue represents the percentage of the CFSE^{lo} population relative to the CD8$^+$ CD45.2$^+$ population. (B) Percentages of the CD45.2$^+$ CD8$^+$ cells that were CFSE^{lo}. (C) The division index of the CD45.2$^+$ CD8$^+$ cells, representing the average number of cell divisions of all the transferred F5 CD8$^+$ T cells. (D) Percentages of the CD45.2$^+$ CD8$^+$ cells that were CD25$^+$. (E) Percentages of CFSE^{lo} CD25$^+$ cells within the CD8$^+$ CD45.2$^+$ population. Results shown in (B-E) are pooled from four independent experiments and are shown as mean ± SEM (WR-ESmini-NT60NP and WR-Full-NT60NP: n = 12; WR-delTK: n = 4).


i.p. 1×10^6 PFU WR-mini-NT60NP, WR-Full-NT60NP or WR-delTK
A
WR-ESmini-NT60NP
WR-Full-NT60NP
WR-delTK
CD25
CFSE
59.3
68.1
54.1
51.3
2.92
9.42
B
% CFSE^lo (of CD45.2^+ CD8^+)
C
Division index
D
% CD25^+ (of CD45.2^+ CD8^+)
E
% CFSE^lo CD25^+ (of CD45.2^+ CD8^+)
WR-ESmini-NT60NP
WR-Full-NT60NP
WR-delTK

5.2.7 Increasing the proteasomal processing of PR8NP fails to elicit a strong PR8NP-366-specific $CD8^+$ T cell response in vivo

The previous data confirm that IAV NP-366 minigenes are less immunogenic than the full-length NP antigens. To investigate the mechanisms responsible, a series of studies was performed.

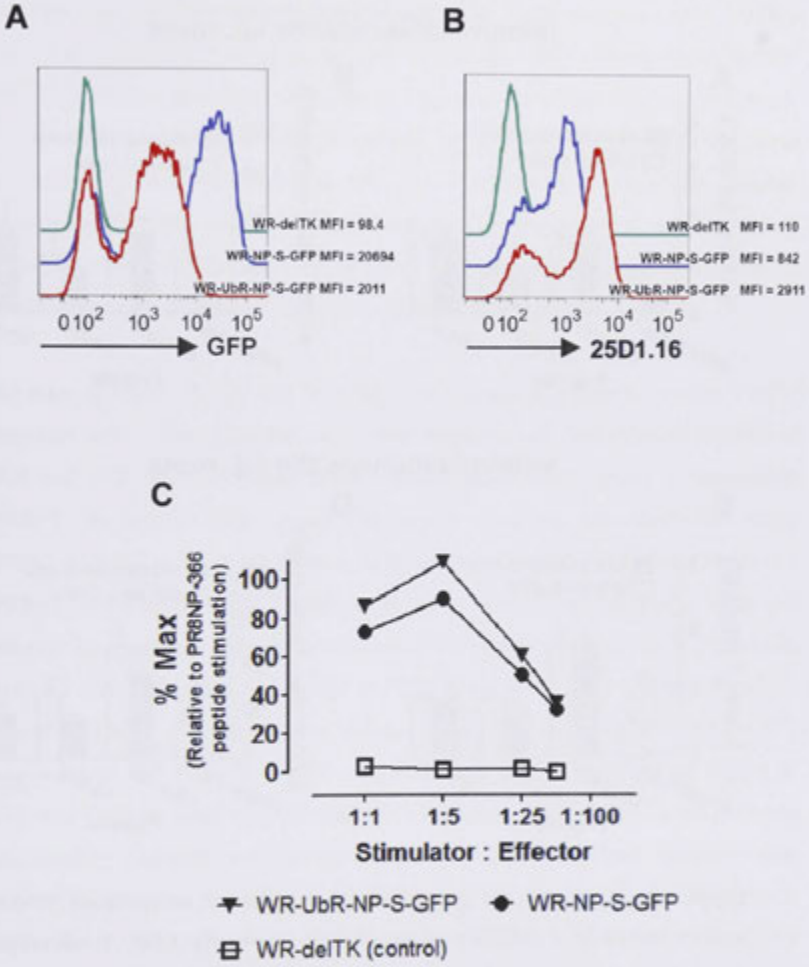
The previous experiments focus on minigene constructs of NP-366 and it seems that the direct presentation efficiency of the IAV NP constructs is inversely proportional to their immunogenicity. Whether enhancing proteasomal processing of full-length IAV NP would affect the priming of the PR8NP-366-specific response remained unknown. Here we took advantage of a set of two recombinant WR viruses expressing a genetically-engineered PR8NP antigen under the control of the VACV p7.5 promoter (Antón et al., 1999; Princiotta et al., 2003). These two viruses are named WR-NP-S-GFP and WR-UbR-NP-S-GFP. The first recombinant have been used in Section 3.2.6. It expresses an antigen called NP-S-GFP which consists of the full-length PR8NP antigen at the amino-terminus, followed by an OVA-257 antigenic peptide and eGFP at the carboxyl-terminus (Antón et al., 1999). WR-UbR-NP-S-GFP expresses a rapidly-degraded version of NP-S-GFP which contains an extra ubiquitin polypeptide and an arginine amino acid residue at the amino-terminus of the NP-S-GFP antigen (Princiotta et al., 2003). The ubiquitin subunit is rapidly cleaved off precisely after the last amino acid residue at its carboxyl-terminus from the fusion antigen by deubiquitinating enzymes soon after translation (Wilkinson et al., 1989). The arginine residue at the amino-terminus of the cleaved antigen will be exposed and it is recognised by E3 ubiquitin ligases to mediate polyubiquitylation of the antigen, which results in rapid degradation of the antigen by the proteasome (Bachmair et al., 1986; Chau et al., 1989; Gonda et al., 1989; Tasaki et al., 2005). The unmodified NP-S-GFP antigen is very stable while UbR-NP-S-GFP is rapidly degraded with a half life of 10 minutes (Princiotta et al., 2003).

WR-NP-S-GFP and WR-UbR-NP-S-GFP have been examined before and it has been shown that the rapidly degraded UbR-NP-S-GFP construct allows better OVA-257/H-$2K^b$ presentation on the surface of infected cells (Princiotta et al., 2003). However, the effect on PR8NP-366 presentation has not been studied. Here

we used the in vitro direct presentation assay described in Section 5.2.1 to measure the direct presentation efficiency of the PR8NP-366 peptide from WR-NP-S-GFP or WR-UbR-NP-S-GFP. Firstly, aliquots of DC2.4 cells infected with WR-NP-S-GFP and WR-UbR-NP-S-GFP for five hours were examined with 25D1.16 antibody and flow cytometric analysis to determine the expression level of GFP and OVA-257/H-2K^b. The results in Figure 5-12A and B demonstrate that DC2.4 cells infected with WR-UbR-NP-S-GFP had lower GFP expression but higher OVA/H-2K^b surface expression when compared to cells infected with WR-NP-S-GFP. This confirms the published studies by Princiotta et al. (2003). Aliquots of the infected DC2.4 cells (as stimulators) were also co-cultured with splenocytes isolated from mouse infected with IAV strain PR8 seven days earlier (as effectors). From this in vitro presentation assay, we found that DC2.4 cells expressing the rapidly degraded UbR-NP-S-GFP induced a slightly highly frequency of IFN-γ-producing CD8$^+$ T cells than the stable NP-S-GFP (Figure 5-12C). A similar result was observed when infected BMDCs or 293-D^b were used as stimulators (data not shown). This suggests that WR-UbR-NP-S-GFP allows effective direct presentation of PR8NP-366 on the infected cells surface.

The immunogenicity of WR-UbR-NP-S-GFP to prime PR8NP-366-specific CD8$^+$ T cell response in vivo was then measured. Similar to the results of the PR8NP-366 minigene constructs, mice immunised with WR-UbR-NP-S-GFP showed a smaller PR8NP-366-specific CD8$^+$ T cell response than mice immunised with WR-NP-S-GFP (Figure 5-13). The difference observed in the i.p. immunisation experiment was not significant because of variations in the responses amongst individual mice (Figure 5-13A and B). In contrast, there was a significant difference between the PR8NP-366-specific CD8$^+$ T cell response, measured as percentage or absolute number, induced by i.d. injection with WR-NP-S-GFP and WR-UbR-NP-S-GFP (Figure 5-13C and D). Taken together with the previous results shown in this chapter, it seems that expressing PR8NP-366 as antigenic constructs that facilitate direct presentation does not effectively induce PR8NP-366-specific CD8$^+$ T cell response in vivo.

Figure 5-12| Expressing PR8NP-366 as a rapidly-degraded construct from WR allows effective direct presentation of the PR8NP-366 peptide. DC2.4 cells were infected with the indicated recombinant WR at a m.o.i. of 5 for 5 h. (A and B) Aliquots of cells were taken and stained with 25D1.16 antibody and were analysed by flow cytometry. Representative histograms illustrate the level of GFP expression (A) and 25D1.16 antibody staining (B) of cells infected with the indicated viruses. (C) Aliquots of the infected cells were also co-cultured with splenocytes from a mouse immunised i.p. with 500 HAU IAV-PR8 seven days before, at different stimulator:effector ratios as indicated. BFA was added 1 h later, followed by another 3 h of co-culturing. ICS was then performed. The overall PR8NP-366-specific $CD8^+$ T cell response of the splenocytes was determined in parallel by standard ICS with synthetic PR8NP-366 peptide. Data show the level of PR8NP-366-specific $CD8^+$ T cell activation by the infected DC2.4 cells, after normalisation to the percentage of maximal potential activation as induced by the synthetic PR8NP-366 peptide. (A, B and C) Results shown are from a single experiment. This whole experiment has been repeated using BMDCs as stimulators and showed similar results. Data in (A) and (B) was confirmed with two independent experiments with DC2.4 cells. Data in (C) was confirmed with an independent experiment with DC2.4 cells. Another experiment using $293D^b$ cells as stimulators also produced similar results.


A
WR-delTK MFI = 98.4
WR-NP-S-GFP MFI = 20694
WR-UbR-NP-S-GFP MFI = 2011
0 10^2 10^3 10^4 10^5
GFP
B
WR-delTK MFI = 110
WR-NP-S-GFP MFI = 842
WR-UbR-NP-S-GFP MFI = 2911
0 10^2 10^3 10^4 10^5
25D1.16
C
% Max
(Relative to PR8NP-366 peptide stimulation)
100
80
60
40
20
0
1:1 1:5 1:25 1:100
Stimulator : Effector
WR-UbR-NP-S-GFP
WR-NP-S-GFP
WR-delTK (control)

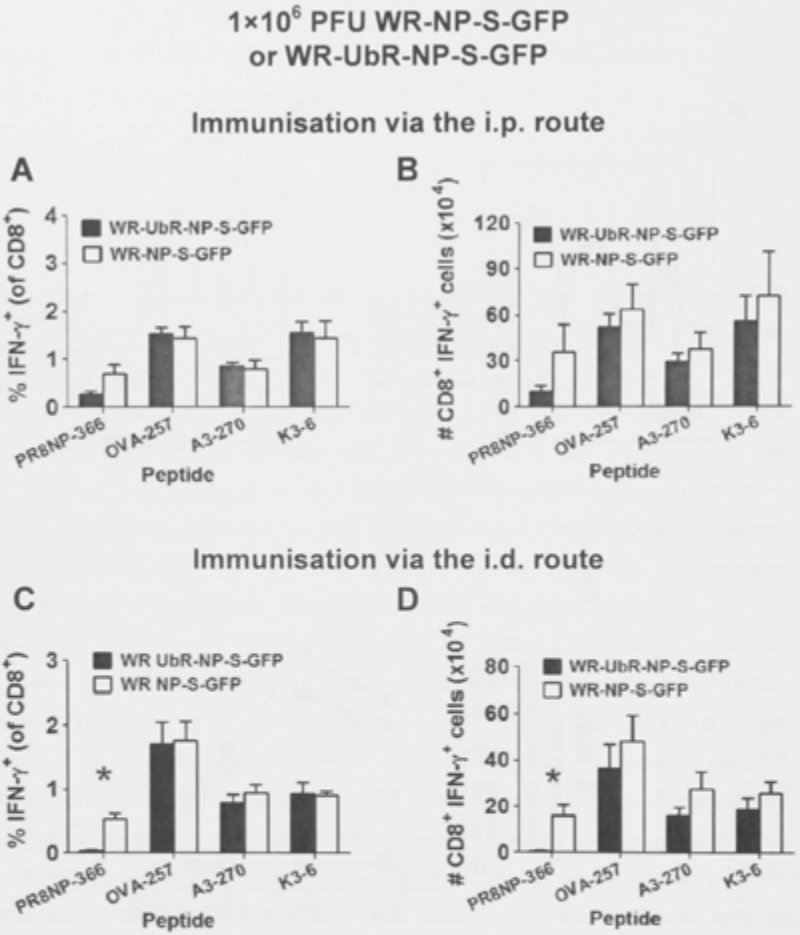


Figure 5-13| Enhancing the proteasomal degradation of recombinant NP-S-GFP antigen results in a reduction of the PR8NP-366-specific $CD8^+$ T cell response. Groups of three mice were immunised with 1×10^6 PFU WR-UbR-NP-S-GFP or WR-NP-S-GFP via i.p. (A and B) or i.d. (C and D) injection. $CD8^+$ T cell responses were determined by ex vivo peptide stimulation and ICS 7 days later. Percentages (A and C) and absolute numbers (B and D) of responding $CD8^+$ T cells to the indicated peptides are shown as mean ± SEM. Data represent results compiled from two independent experiments (n = 6 for each immunisation route). * denotes statistical significance ($p < 0.05$).

5.2.8 There is no evidence to support cross presentation of PR8NP-366 from VACV-infected cells

The above results show that PR8NP-366 expressed as directly presented constructs do not allow optimal inductions of $CD8^+$ T cell responses. One possible reason is that PR8NP-366-specific $CD8^+$ T cells are cross primed during VACV infection. To test this hypothesis, the in vivo cross presentation assay was used. 293A cells were infected with WR-ESmini-PR8NP or WR-Full-PR8NP. These infected cells were heat treated at 60°C for 60 min to inactivate all the residual viruses and the cells were then injected i.p. into mice at a dose of 1×10^7 infected cells per mouse. Seven days later, $CD8^+$ T cell responses against several native VACV peptides and PR8NP-366 were determined in spleens.

As seen in Figure 5-14A and B, $CD8^+$ T cell responses against several VACV peptides were induced by the 293A cells infected with WR-ESmini-PR8NP or WR-Full-PR8NP. By contrast, these infected cells did not prime a measurable PR8NP-366-specific $CD8^+$ T cell response in mice. Another experiment using 2×10^7 infected cells to immunise individual mice was conducted. However, PR8NP-366-specific $CD8^+$ T cell responses were still not detectable (data not shown). In a separate experiment in which mice were immunised with 293A cells infected with either WR-NP-S-GFP or WR-UbR-NP-S-GFP, a higher OVA-257-specific $CD8^+$ T cell response was induced by cells infected with WR-NP-S-GFP, compared to WR-UbR-NP-S-GFP (the difference was not significant; Figure 5-14C and D). This confirmed the findings by Norbury, et al. (2004) which showed that rapidly degraded antigens cannot be cross presented from infected cells. However, the same recombinant NP-S-GFP antigen available in the WR-NP-S-GFP-infected cells did not induce any detectable PR8NP-366-specific $CD8^+$ T cell response (Figure 5-14C and D). In summary, we found no evidence suggesting that PR8NP-366 specific $CD8^+$ T cells are cross primed from VACV-infected cells.

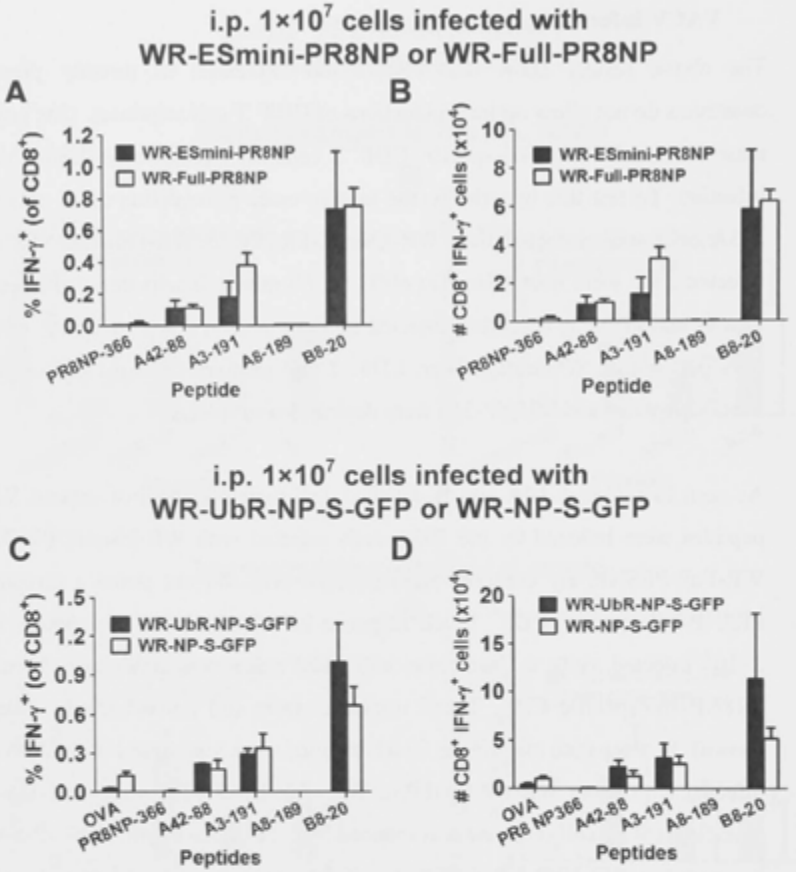


Figure 5-14| The PR8NP-366-specific CD8$^+$ T cell response cannot be cross primed from cells infected with PR8NP-366-expressing WR. 293A cells were infected with the indicated viruses at a moi of 5 for 6 h, followed by heat treatment at 60°C for 60 min. Groups of three mice were then i.p. immunised with 1×10^7 of these infected, heat-treated cells. Seven days later, antigen-specific CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells responding to the indicated peptides are shown as means ± SEM. (A and B) Data represent results from one experiment (n = 3). Another independent experiment in which mice were immunised with 2×10^7 infected, heat-treated cells showed similar results. (C and D) Data represent results from one single experiment (WR-UbR-NP-S-GFP: n = 2; WR-NP-S-GFP: n = 3). * denotes statistical significance ($p < 0.05$).

5.2.9 BMDCs pulsed with PR8NP-NP-366 and a $CD4^+$ T cell epitope peptide does not elicit a PR8NP-NP-366-specific $CD8^+$ T cell response

A requirement of $CD4^+$ T cell help for a $CD8^+$ T cell response can be epitope-specific, that is only some epitope-specific $CD8^+$ T cell responses require $CD4^+$ T cell help (Snyder et al., 2009; Reiser et al., 2011). This might reflect a scenario in which DCs expressing both $CD4^+$ and $CD8^+$ T cell epitopes can firstly be activated by $CD4^+$ T cells, which are then able to prime $CD8^+$ T cell responses (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Smith et al., 2004; Beuneu et al., 2006). Alternatively, activated $CD4^+$ T cells might be attracted by DCs in an antigen-specific manner to provide help directly to the activating $CD8^+$ T cells (Bourgeois et al., 2002). In the classical pathway, peptides bound on MHC-II are processed from exogenous antigens in the endosomes of APCs (reviewed in Jensen, 2007; Blum et al., 2013). However, it is also known that some endogenously synthesised antigens can be processed within APCs to liberate peptides for MHC-II presentation (Eisenlohr and Hackett, 1989; Jaraquemada et al., 1990; Loss et al., 1993; Rudensky et al., 1994; Oxenius et al., 1995; Dani et al., 2004). This might be the pathway that allows both MHC-I- and MHC-II-restricted peptides from the same antigen/pathogen to be displayed on the same DCs.

IAV NP contains several $CD4^+$ T cell epitopes specific to MHC-II I-A^b molecules of C57Bl/6 mice (Gao et al., 1990b; Crowe et al., 2006). However, the minigene constructs examined in this thesis do not contain any $CD4^+$ T cell epitopes. In addition, endogenously-synthesised antigens that are rapidly degraded by the proteasome do not allow effective MHC-II presentation (Guéguen and Long, 1996). Therefore, a special requirement for direct $CD4^+$ T cell help might explain why minigenes and rapidly-degraded constructs were less able to induce $CD8^+$ T cell responses against IAV NP-366 peptides in vivo. However, this effect needs be specific to the IAV NP-366 minigene when expressed from VACV. This is because $CD8^+$ T cell response against IAV NP-366 can be induced in $CD4^+$-T-cell-deficient mice after primary IAV infection (Belz et al., 2002). In addition, minigenes of other antigens were highly immunogenic when expressed from WR, as shown in Chapter 4. It should be noted that there are $CD4^+$ T cell epitopes available from VACV proteins (Jing et al., 2007; Moutaftsi et al., 2007). Although

it is reasonable to assume that they are presented on the same DCs with together the $CD8^+$ T cell epitopes, it has not been formally examined.

To test this hypothesis, peptide-pulsed BMDCs were used as immunogens. Here, BMDCs generated from C57Bl/6 mice were pulsed with a synthetic $CD8^+$ T cell epitope peptide together with the HBcAg-128 peptide. The HBcAg-128 peptide is a $CD4^+$ T cell epitope from the hepatitis B virus core antigen, restricted to MHC-II $I\text{-}A^b$ (sequence TPPAYRPPNAPIL; Milich et al., 1988; Alexander et al., 1994). After peptide pulsing, BMDCs were injected i.v. into mice and $CD8^+$ T cell responses were measured seven days later. This priming assay allowed induction of $CD8^+$ T cells against VACV A47-171 peptide if BMDCs were pulsed with a synthetic A47-171 peptide (Figure 5-15). In contrast, an anti-PR8NP-366 $CD8^+$ T cell response was not induced when the PR8NP-366 peptide was used for pulsing BMDCs (Figure 5-15), implying that an addition of $CD4^+$ T cell epitope on the PR8NP-366-presenting DCs might not be enough for priming $CD8^+$ T cell responses against PR8NP-366.

5.2.10 The sub-cellular localisation of full-length PR8NP does not influence its immunogenicity

As discussed in the introduction of this chapter, IAV NP can be found within the nucleus of IAV-infected cells. Similarly, when PR8NP is expressed from VACV, it is localised in the nucleus (Antón et al., 1999; Golovina et al., 2002). It has been suggested that proteasomes within the nucleus (Reits et al., 2003, Reits et al., 1997) can process nuclear antigens for MHC-I presentation (Antón et al., 1999). The nuclear localisation of the full-length IAV NP antigen might influence how it is processed. Alternatively, the antigen might be post-translationally modified within the nucleus and affect the antigenicity of the presented IAV NP-366 peptide.

To investigate if the nuclear localisation property of PR8NP influences the PR8NP-366-specific $CD8^+$ T cell response, two WR recombinants expressing modified forms of PR8NP were tested. The first recombinant, known as WR-cyto-PR8NP, expresses an antigen with the amino acid residues 13-498 of PR8NP,

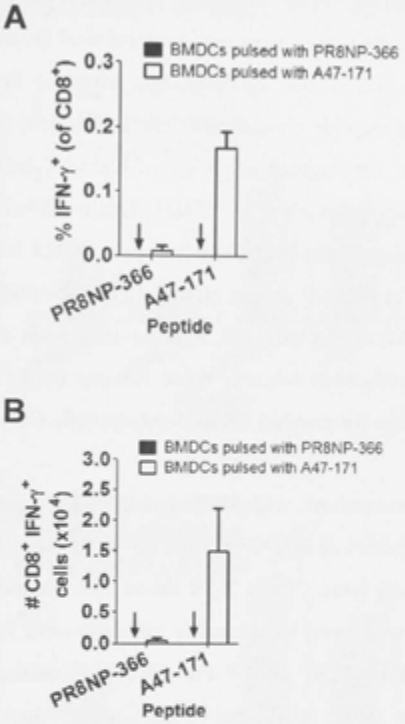


Figure 5-15| BMDCs pulsed with PR8NP-NP-366 and a $CD4^+$ T cell epitope peptide fail to prime PR8NP-NP-366-specific $CD8^+$ T cells. In vitro-generated BMDCs were pulsed with the synthetic MHC-II-binding HBcAg-128 peptide together with the PR8NP-366 or VACV A47-171 peptides. These pulsed cells were injected i.p. into groups of two mice (2×10^6 cells/mouse). Seven days later, $CD8^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A) and absolute numbers (B) of $CD8^+$ T cells specific to PR8NP-366 and A47-171 are shown as mean ± SEM from a single experiment (n = 2). Arrows illustrate the absence of responses induced by BMDCs pulsed with PR8NP-366. This experiment has only been performed once.

excluding the nuclear localisation signal corresponding to the amino acid sequence 1-12 of the full-length PR8NP (Golovina et al., 2002). The loss of this nuclear localisation signal sequence prevents PR8NP from entering into the nucleus and results in the cytoplasmic retention of the modified PR8NP (Golovina et al., 2002). The second recombinant, which is known as WR-ES-PR8NP, expresses a polypeptide consisting of the amino acid residues 13-498 of PR8NP tagged with an ER-targeted sequence of the IAV hemagglutinin protein at the amino-terminus (Golovina et al., 2002). This modification of PR8NP causes the antigen to localise to the ER (Golovina et al., 2002). It has been shown that these modifications to PR8NP do not alter the PR8NP-366/H-2D^b presentation on the surface of VACV-infected cells in vitro (Golovina et al., 2002). However, it remained undetermined whether these variants might affect how the antigen is processed in vivo for priming PR8NP-366-specific CD8$^+$ T cell responses.

Mice were immunised with WR recombinants expressing the wildtype or localisation variants of PR8NP via the i.p. route and CD8$^+$ T cell responses were measured 7 days later. Figure 5-16 shows that the induced PR8NP-366-specific CD8$^+$ T cell responses were similar amongst mice immunised with WR-cyto-PR8NP, WR-ES-PR8NP or WR-Full-PR8NP. This suggests that the sub-cellular location of the stable full-length PR8NP antigen does not affect the priming of PR8NP-366-specific CD8$^+$ T cell in vivo. This also implies that wildtype PR8NP is not exclusively processed within the nucleus to liberate PR8NP-366 for MHC-I presentation, confirming the published in vitro studies (Golovina et al., 2002). Together with results from Figure 5-13, the data imply that a stable full-length protein may be required to prime an effective CD8$^+$ T cell response against PR8NP-366 when expressed from VACV.

5.2.11 The serine residue at position 2 of the PR8NP-366 peptide is not phosphorylated

In addition to presenting natural peptides on MHC-I, peptides carrying post-translational modification can also bind to MHC-I to induce CD8$^+$ T cell responses (Haurum et al., 1994; Skipper et al., 1996; Meadows et al., 1997; Haurum et al., 1999; Zarling et al., 2000; Zarling et al., 2006). One such

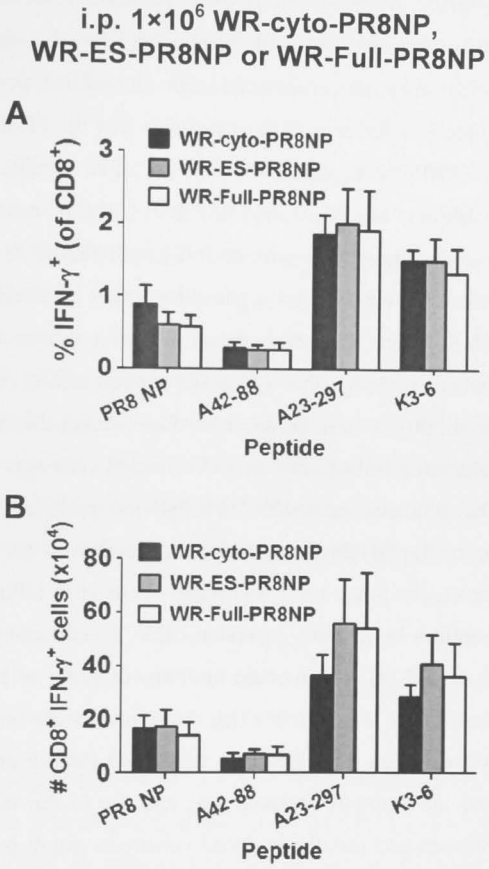


Figure 5-16| The sub-cellular localisation of the full-length PR8NP antigen does not determine its ability to prime anti-PR8NP-366 $CD8^+$ T cells when expressed from WR. Groups of three mice were immunised with the indicated recombinant WR (1×10^6 PFU) via the i.p. route. Seven days later, $CD8^+$ T cell responses were determined by ex vivo peptide stimulation and ICS and the results are shown as percentages (A) or absolute numbers (B) of $CD8^+$ T cells specific to the peptides indicated. Data shown represent results compiled from two independent experiments (mean ± SEM; n = 6).

modification is phosphorylation (Zarling et al., 2000; Zarling et al., 2006), which can occur on serine, threonine, or tyrosine residues (reviewed in Ubersax and Ferrell Jr, 2007). Due to the specificity of $CD8^+$ T cells, responses specific to modified peptides may not cross-react with unmodified peptides. As seen in Section 5.1, there is a serine residue at position 2 of the PR8NP-366 peptide (as well as the NT60NP-366 peptide). With the use of two online programs, namely NetPhos 2.0[1] (Blom et al., 1999) and DISPHOS 1.3[2] (Iakoucheva et al., 2004), to predict putative phosphorylation sites on full-length PR8NP, it was found that the serine residue on PR8NP-366 has a phosphorylation potential above the cut-off values of each software. However, it has not been shown whether this serine residue represents a real phosphorylation site experimentally (Kistner et al., 1989; Hutchinson et al., 2012). In addition, it has been shown that the natural PR8NP-366 peptide extracted from $H\text{-}2D^b$ of IAV-infected cells was eluted in the same fraction as the synthetic unmodified PR8NP-366 peptide using reverse-phase high-performance liquid chromatography (Rotzschke et al., 1990; Falk et al., 1991a). However, the assay used was based on in vitro killing of APCs pulsed with eluted peptides by in vitro-generated $CD8^+$ T cell clones (Rotzschke et al., 1990; Falk et al., 1991a), which could limit any diversity of peptides that can be identified. Therefore, it was possible that the serine residue on PR8NP-366 might be phosphorylated when expressed as a full-length PR8NP antigen from VACV strain WR and the response induced was specific to the modified peptide. In contrast, minigenes and rapidly-degraded constructs might not be recognised as substrates for phosphorylation and therefore no responses against PR8NP-366 could be induced. If this is the case, the unmodified peptide was able to re-stimulate the response specific to the phosphorylated PR8NP-366 ex vivo in the ICS assay only when an unphysiologically high, not low, concentration of the peptide was used.

To examine this hypothesis, we examined how well a synthetic peptide of amino acid sequence ASNENMETM with the serine phosphorylated can be used to stimulate polyclonal PR8NP-366-specific $CD8^+$ T cells ex vivo. In brief,

[1] NetPhos 2.0. Website: http://www.cbs.dtu.dk/services/NetPhos/
[2] DISPHOS 1.3. Website: http://www.dabi.temple.edu/disphos/pred.html

splenocytes isolated from a mouse immunised with WR-Full-PR8NP seven days earlier were ex vivo stimulated with serial dilutions of the unmodified PR8NP-366 peptide or the serine-phosphorylated (PR8NP-366-pS) peptide, followed by ICS. Stimulation with either peptide at a concentration of 10^{-6} M induced a similar proportion of $CD8^+$ T cells from the WR-Full-PR8NP-immunised mouse to produce IFN-γ (Figure 5-17). However, when the peptide concentration was diluted, the potency of the phosphorylated PR8NP-366 peptide to stimulate the WR-Full-PR8NP-immune $CD8^+$ T cells decreased rapidly and it was below the detection limit when 10^{-9} M was used. In comparison, the unmodified PR8NP-366 peptide was still able to induce IFN-γ expression from the $CD8^+$ T cells when a concentration of 10^{-11}M was used (Figure 5-17). This study suggests that the PR8NP-366-specific $CD8^+$ T cells induced by the full-length PR8NP expressed from WR does not recognise the serine-phosphorylated PR8NP-366 peptide. Therefore, the naturally-occurring PR8NP-366 peptide on MHC-I during in vivo immunisation is not phosphorylated at the serine residue at the 2nd position of the peptide.

5.3 Discussion

Results in this chapter show that IAV NP expressed as constructs to facilitate direct presentation were unable to prime a potent $CD8^+$ T cell response against the NP-366 epitope. This phenomenon occurs for two well-studied variants of the epitope, PR8NP-366 and NT60NP-366. There are several studies showing that minigene constructs of certain antigens do not induce $CD8^+$ T cell immunity in mouse models when expressed from VACV strain WR. However, in most cases, the full-length antigens are also unable to prime $CD8^+$ T cell responses and the lack of immunogenicity was not because of a defect of minigene constructs.

The first example is the antigenic peptide from sequence 209-217 (ITDQVPFSV) of the tumour-associated antigen gp100 (Kawakami et al., 1995), which was not immunogenic when expressed as an ER-targeted minigene or as a full-length antigen from WR (Irvine et al., 1999). The lack of immunogenicity of this peptide was attributed to its relatively low affinity for its restricted human MHC-I LA-

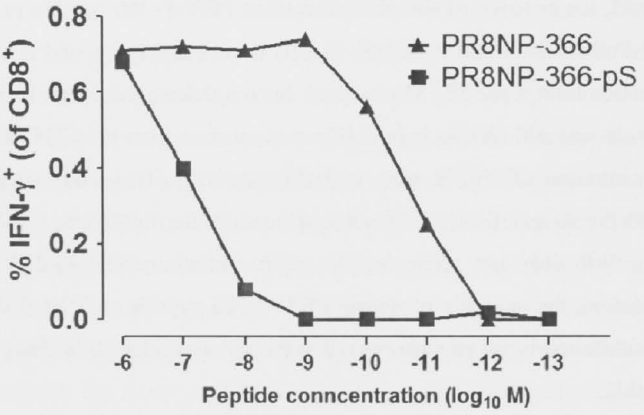


Figure 5-17| The recognised PR8NP-366 peptide is not phosphorylated when expressed as a full-length antigen from WR. A mouse was immunised with 1×10^6 PFU WR-Full-PR8NP. Seven days later, splenocytes from the immunised mouse were stimulated ex vivo with either an unmodified PR8NP-366 peptide or with a modified version of the peptide carrying a phosphorylated serine residue (PR8NP-366-pS; amino acid sequence: A[pS]NENMETM) at the concentration indicated. The results are shown as percentages of $CD8^+$ T cells that produced IFN-γ after peptide stimulation. Data are representative of two independent experiments.

A*0201 molecule (Parkhurst et al., 1996; Irvine et al., 1999). Enhancing the binding affinity by modifying the anchor sequence in this peptide and expressing it as an ER-targeted minigene from WR resulted in an induction of a robust $CD8^+$ T cell response against this peptide (Irvine et al., 1999). Another similar example was the antigenic peptide with amino acid sequence RSPWFTTL from the envelope protein of Friend/Moloney/Rauscher murine leukemia retroviruses (Coppola and Green, 1994; Sijts et al., 1994). This peptide binds to its restricted MHC-I H-$2K^b$ with a similar affinity and dissociation rate as another related antigenic peptide with sequence KSPWFTTL from AKR/Gross murine leukemia retroviruses (Sijts et al., 1994; Kim and Green, 1998). However in C57Bl/6 mice, only the KSPWFTTL peptide, but not RSPWFTTL, was able to induce $CD8^+$ T cell responses when expressed as a full-length or as a minigene construct from WR (Coppola and Green, 1994; Coppola et al., 1995; Kim and Green, 1998). The lack of immunogenicity of the RSPWFTTL-containing construct expressed from WR is because there are no naive $CD8^+$ T cells recognising this peptide in wildtype mice (Kim et al., 2000). As the last example, two studies have examined WR recombinants expressing a model tumour-associated antigen P1A (Restifo et al., 1995; Imai et al., 2010). P1A contains a H-$2L^d$-restricted antigenic peptide known as P1A-35 with the amino acid sequence LPYLGWLVF (Lethé et al., 1992). Full-length P1A antigen expressed from WR was more immunogenic, even though both constructs were directly presented in vitro with a similar efficiency (Irvine et al., 1995; Restifo et al., 1995). However, an ER-targeted P1A-35 minigene, which also allowed effective direct presentation in vitro, was highly immunogenic, inducing a greater $CD8^+$ T cell response than the full-length antigen (Irvine et al., 1995; Restifo et al., 1995). It was hypothesised that the reduced immunogenicity of the cytosolic minigene was caused by a reduction of antigen presentation by the relevant APC in vivo, probably due to degradation of peptide in the cytosol or relatively poor transport by TAP in vivo (Irvine et al., 1995; Restifo et al., 1995). However, no experiments were performed to test these hypotheses.

Our findings that PR8NP-366 and NT60NP-366 were not immunogenic when expressed as constructs from WR to facilitate direct presentation is clearly different from the examples mentioned above. Besides the minigene and rapidly-

degraded constructs examined in this chapter, PR8NP-366 expressed as part of a string of multiple antigenic peptides, known as a polytope, from VACV strain WR also failed to induce any detectable PR8NP-366-specific $CD8^+$ T cell response with an ex vivo peptide stimulation assay (Flesch et al., 2010). Our results showed that WR expressing ER-targeted PR8NP-366 minigene induced a secondary expansion of memory PR8NP-366 specific $CD8^+$ T cells in mice previously infected with IAV strain PR8 although the expansion level was significantly less than WR expressing the full-length PR8NP antigen. This suggested that the anti-PR8NP-366 $CD8^+$ T cell response induced by the ER-targeted minigene was poor, but not absent. Our data with UbR-NP-S-GFP suggests that increasing the proteasomal processing rate of PR8NP did not translate to an improved ability to induce PR8NP-366 responses in vivo. However, the PR8NP-366 peptide from UbR-NP-S-GFP can be readily presented in vitro, indicating a dissociation of in vitro presentation from the in vivo immunogenicity of this $CD8^+$ T cell epitope. One possibility is that over-stimulation of NP-366-specific $CD8^+$ T cells with a high level of peptide/MHC-I complexes may be detrimental to the immune outcome. Other than the NP-366 peptide, IAV NP also contains a H-$2K^d$-binding peptide in position 147-155 (NP-147, sequence TYQRTRALV; Falk et al., 1991a). It has been shown that generation of this peptide for MHC-I presentation in vitro from cells infected with WR expressing full-length IAV NP was either not affected or even enhanced in the presence of proteasome inhibitors (Yellen-Shaw et al., 1997; Antón et al., 1998). Interestingly, enhancing proteasomal degradation of IAV NP by adding an ubiquitin and an arginine residue at the N terminus of the IAV NP antigen did not inhibit MHC-I presentation of NP-147 (Golovina et al., 2005). It was later suggested that the enhanced presentation of NP-147 on cells treated with proteasome inhibitors was because of a change in the proteasomal cleavage specificity (Wherry et al., 2006). Together with the data of IAV NP-366 shown in this chapter, it clearly suggests that our understanding of antigen processing, antigen presentation level and immunogenicity is still limited.,

Experiments with F5 TCR transgenic $CD8^+$ T cells demonstrate that the NT60NP-366 minigene expressed from WR induced a smaller F5 $CD8^+$ T cell response than full-length NT60NP starting from six days post immunisation. However, this minigene induced similar levels of early activation (CD69 and CD25) and

proliferation (CFSE dilution) of F5 $CD8^+$ T cells as the full-length NT60NP antigen two days after immunisation. The magnitude of a $CD8^+$ T cell response is determined by both the proliferation and the survival of the antigen-specific $CD8^+$ T cells. CD25 expression and cell proliferation of the F5 $CD8^+$ T cells were only measured two days post immunisation in this study. It is possible that the NT60NP-366 minigene fails to maintain CD25 expression at later stages of the priming phase. The kinetics of CD25 expression and F5 $CD8^+$ T cell proliferation should be examined. In addition, it is likely that the ER-targeted NT60NP-366 minigene fails to promote survival of the responding $CD8^+$ T cells. Expression of pro-survival proteins, including Bcl-2 or B-cell lymphoma-extra large (Bcl-X_L) (Nakayama et al., 1994, Boise et al., 1995, Grayson et al., 2000, Veis et al., 1993), and pro-apoptotic proteins, such as Bim (O'Connor et al., 1998, Bouillet et al., 1999), can influence the survival of activated $CD8^+$ T cells in vivo (Kurtulus et al., 2011, Hildeman et al., 2002, Pellegrini et al., 2003, Lee et al., 2002, Song et al., 2005). The expression of these genes should be determined in future studies to determine whether and how the survival of NT60NP-366-specific $CD8^+$ T cells is influenced by priming with the ER-targeted NT60NP-366 minigene. The studies with the F5 $CD8^+$ T cells also suggested that any differences occurring early after immunisation are subtle, but these changes ultimately suppress optimal induction of a NT60NP-366-specific $CD8^+$ T cell response.

Because only stable antigens can be used as substrates for cross presentation (Norbury et al., 2004; Shen and Rock, 2004; Wolkers et al., 2004), it might explain why antigenic constructs that facilitate direct presentation of PR8NP-366 were not immunogenic in vivo. However, we did not detect cross priming of PR8NP-366-specific $CD8^+$ T cells in mice immunised with MHC-I mismatched cells infected with WR expressing stable PR8NP antigens. As discussed previously in Section 3.3, one major limitation of this assay is that it does not necessarily represent the natural cross priming mechanism for all antigens. However, it should be noted that IAV-infected cells have been shown to cross prime PR8NP-366-specific $CD8^+$ T cells (Chen et al., 2004; Lev et al., 2009). Similarly, MHC-I-deficient RMA-S cells stably expressing a recombinant GFP construct containing the NT60NP-366 peptide could cross prime NT60NP-366-specific $CD8^+$ T cells in vivo (Wolkers et al., 2001). This suggests that the IAV

NP antigen can be cross presented from antigen donor cells. Therefore, the absence of IAV NP cross presentation from VACV-infected cells as seen in our studies is likely to be a result of VACV infection. One possible explanation is that the vast amount of VACV antigens expressed within infected cells may influence the cross presentation of NP in vivo.

Dependence on $CD4^+$ T cell help for priming optimal $CD8^+$ T cells can be epitope-specific (Snyder et al., 2009; Reiser et al., 2011). Priming PR8NP-366-specific $CD8^+$ T cells might require such $CD4^+$ T cell help in vivo. However, we did not detect any measureable response in mice primed with BMDCs with PR8NP-366 and a $CD4^+$ T cell epitope peptide. One limitation of the priming strategy used in Figure 5-15 is that it does not completely recapitulate the priming mechanisms that occur during VACV immunisation. In addition, BMDCs used for immunisation were pulsed with high concentrations of peptides and this might reduce the level of the response induced in the immunised mice. BMDCs pulsed with lower concentrations of peptides could be tested in further studies. An alternative explanation is that the induction of a PR8NP-366-specific $CD8^+$ T cell response may require homologous help from $CD4^+$ T cells specific to peptides available on the NP antigen itself, but not by other heterologous epitopes. A similar requirement for a homologous $CD4^+$ T cell epitope for priming functional $CD8^+$ T cells has been reported (Sabarth et al., 2010). The reasons for this were not explored in that study. One strategy that could be used here is to generate a recombinant WR that expresses a transgene encoding the PR8NP-366 minigene together with a recombinant full-length NP antigen with the PR8NP-366 peptide deleted, separated by an internal ribosome entry site (Jang et al., 1988; Pelletier and Sonenberg, 1988). DCs infected with this virus should express both the PR8NP-366 peptide and homologous $CD4^+$ T cell epitopes from PR8NP for MHC-I and MHC-II presentation respectively. The use of an internal ribosome entry site is essential because it ensures that both antigenic constructs will be expressed within the same cells, without influencing the characteristics of the individual antigens, which would occur if the antigens were expressed as a single fusion protein.

A theory that IAV NP-366 peptides expressed from directly-presented constructs might be slightly different from those expressed from full-length stable antigens was also investigated. Using a mouse infection model with LM expressing OVA-257 variant peptides, it has been shown that the OVA-257 variant peptides with lower affinity to the TCR of OT-I cells still allowed early activation and proliferation of the OT-I cells in vivo (Zehn et al., 2009). However, further expansion of the activated OT-I cells were not sustained by the weak TCR-peptide/MHC-I interactions (Zehn et al., 2009). These results were similar to our results shown in this chapter, in which no significant differences were detected between the early activation and proliferation of F5 $CD8^+$ T cells induced by WR-ESmini-NT60NP or WR-Full-NT60NP. Recently, a report has demonstrated that the low affinity OVA-257 variant peptides limited the up-regulation of transcription factor interferon regulatory factor 4 (IRF4) in OT-I cells (Man et al., 2013). The lower expression of IRF4 reduced metabolic functions of the activating $CD8^+$ T cells and resulted in a decrease in the magnitude of the $CD8^+$ T cell response induced (Man et al., 2013). The role of IRF4 in our models should be investigated. This may provide a clue if the presented peptide is somehow modified. In this thesis, two possible mechanisms which might affect the TCR affinity of the presented IAV NP-366 peptide were examined by focusing on the PR8NP-366 peptide. Firstly, the effect of the nuclear localisation of PR8NP on its immunogenicity was tested. Here we hypothesised that the nuclear localisation of PR8NP might influence the processing of the antigen for MHC-I presentation. Alternatively, the presented PR8NP-366 peptide may be modified post-translationally within the nucleus. However, our experiments revealed that the nuclear localisation of NP does not influence its ability to induce PR8NP-366-specific $CD8^+$ T cells in vivo. Secondly, we have formally ruled out the possibility that the serine residue at position 2 on PR8NP-366 was phosphorylated when presented in vivo for $CD8^+$ T cell priming.

Another possible modification of the presented NP-366 peptide that may occur is oxidation of the methionine residues. Methionine contains a thioether side chain, which consists of a sulphur atom. The sulphur atom can be oxidised to generate methionine sulfoxide by oxidants in vitro and in vivo (Vogt, 1995; Ghesquière et al., 2011). There are two methionine residues on the PR8NP-366 and NT60NP-

366 peptides, one in position 6 and one in position 9. The structure of PR8NP-366 peptide bound on H-2D^b has been solved by X-ray crystallography (Young et al., 1994) and it allows us to deduce the effects of methionine oxidation on the overall peptide/MHC-I structure. Firstly, the methionine in position 9 is one of the major anchor residues and is important for the H-2D^b binding by forming hydrogen bonds with three amino acid residues of H-2D^b (Young et al., 1994). Oxidation of the methionine in this position may be detrimental to the MHC-I affinity of the peptide. For the methionine residue in position 6, its side chain is exposed to the surface of the top of the PR8NP-366/H-2D^b structure and forms a hydrogen bond with the imidazole side chain of the histidine at position 155 of H-2D^b (Young et al., 1994; Turner et al., 2005). Structures of H-2D^b bound with PR8NP-366 with the methionine at position 6 replaced with different amino acid residues have been analysed (Kedzierska et al., 2008; Valkenburg et al., 2013). All amino acid substitutions resulted in various degrees of conformational changes to the bound peptide and the H-2D^b molecule surrounding the modified residue (Kedzierska et al., 2008; Valkenburg et al., 2013). Importantly, amino acid substitutions at position 6 of PR8NP-366 expressed from IAV alters the TCR repertoires of the responding CD8$^+$ T cells in vivo and thereby influences the primed CD8$^+$ T cell responses as measured during acute infection (Kedzierska et al., 2008; Valkenburg et al., 2013). We hypothesise that oxidation of methionine in position 6 may result in a subtle change in the peptide/MHC-I structure. All peptide stocks used in this thesis were prepared in DMSO before further dilution in culture media. It has been shown that methionine and cysteine can be oxidised in DMSO under certain conditions (Shechter, 1986). Thus, it is possible that peptides used in ICS and DimerX assays might have already been oxidised. If this assumption is true, the difference between the antigenicity of the oxidised and unoxidised peptides would be subtle because the DimerX binding and the performance of peptides prepared in DMSO in ex vivo ICS assays seemed not to be affected. Such a modification may subtly reduce the in vivo priming of CD8$^+$ T cells early after immunisation, but eventually greatly affect the immunogenicity at the peak of the response.

The oxidation state of amino acid residues on IAV NP has not been examined before. NP expressed as a native full-length antigen may be protected from

oxidation while the NP-366 peptide may be prone to oxidisation when expressed from recombinant constructs that facilitate direct presentation. It has been shown that an antigenic peptide containing a cysteine residue expressed as a minigene from VACV WR can be oxidised and form a disulfide bond with a free cysteine residue (known as cysteinylation) within infected cells (Chen et al., 1999). Importantly, this cysteinylated peptide can be presented onto MHC-I (Chen et al., 1999). We attempted to address whether unoxidised PR8NP-366 peptide could prime a $CD8^+$ T cell response in vivo. Here mice were immunised with BMDCs pulsed with the HBcAg-128 $CD4^+$ T cell epitope peptide together with PR8NP-366 peptide prepared in water, which should prevent methionine oxidation. No detectable $CD8^+$ T cell response against PR8NP-366 was induced in this experiment (data not shown). The main limitation of this experiment was that the oxidation state of the peptide stocks prepared in DMSO or water was not determined. In addition, it remains unclear whether the peptide would become oxidised during peptide-pulsing. A new experimental approach is required here. To formally determine whether the chemical identity of the presented PR8NP-366, mass spectrometric analysis of PR8NP-366 peptides presented on DC2.4 cells infected with WR-ESmini-PR8NP or WR-Full-PR8NP is being pursued (through collaboration with Dr. Nathan Croft and Prof. Anthony Purcell from the University of Melbourne, Australia).

In summary, results in this chapter show that $CD8^+$ T cell responses specific to IAV NP-366 cannot be induced by IAV NP constructs that promote direct presentation when expressed from VACV strain WR. However, the mechanism involved remains elusive.

Chapter 6 The application of heat-inactivated vaccinia virus a recombinant $CD8^+$ T cell vaccine

6.1 Introduction

As seen in Chapter 3, antigens from VACV-infected MHC-I mismatched cells can cross prime $CD8^+$ T cells against multiple native VACV peptides. Interestingly, some of these peptides originate from antigens that are found within VACV virions. These antigens include A3, a VACV major core protein, A42, a profilin-like protein, and J3, a multi-functional protein involved in VACV gene transcription (Jensen et al., 1996; Chung et al., 2006; Yoder et al., 2006; Resch et al., 2007). It is possible that some VACV particles remained associated on the surface of the infected cells and their role in $CD8^+$ T cell priming has not been determined.

Cross presentation of antigens from virion particles has been observed for IAV. Using in vitro systems, it has been shown that APCs co-cultured with UV-inactivated or heat-inactivated IAV were recognised by anti-IAV $CD8^+$ T cells (Hosaka et al., 1985; Bender et al., 1995). Another study reported a similar finding with UV-inactivated IAV, but only when the virus was subject to further heat inactivation (Yewdell et al., 1988). Importantly, the induction of MHC-I presentation on cells pulsed with inactivated IAV does not require viral protein synthesis (Hosaka et al., 1985; Yewdell et al., 1988; Bender et al., 1995), demonstrating that the source of the presented peptides on MHC-I is the inactivated virus particles. In vivo cross priming of $CD8^+$ T cells of antigens from exogenous NP fractionated from IAV virions and heat-inactivated IAV has also been reported (Wraith and Askonas, 1985; Cho et al., 2003).

Besides using live recombinant viruses to induce immunity against foreign antigens, virus-like particles have also been used as recombinant vaccines. In general, virus-like particles are microscopic structures formed by multiple copies of one or more viral proteins from a single virus (reviewed in Roldão et al., 2010). These particles do not contain any viral genomes and so they are not infectious. Several vaccines based on virus-like particle technology are commercially available, including the human Papillomavirus vaccine Gardasil (reviewed in Roldão et al., 2010). Vaccines based on chimeric virus-like particles have also been studied (e.g. Sedlik et al., 1997; Neirynck et al., 1999). These particles

contain antigens of different origins that are tagged to the components on the virus-like particles, allowing induction of immune responses against the foreign antigens (Moron et al., 2004; Roldão et al., 2010). Importantly, virus-like particles have been shown to cross prime $CD8^+$ T cell responses in vivo (Sedlik et al., 1997; Win et al., 2011; Hemann et al., 2013). Further studies have shown that virus-like particles can be taken up by DCs readily, resulting in DC activation and allowing antigens on the particles to be processed for MHC-I presentation (Ruedl et al., 2002; Fausch et al., 2003; Morón et al., 2003; Barth et al., 2005; Song et al., 2010; Win et al., 2011). These data suggest that antigens available on virions or on virus-like particles induce $CD8^+$ T cell responses via cross priming, without the requirement for nascent protein synthesis.

In this chapter, the ability of heat-inactivated VACV virion particles to induce $CD8^+$ T cell responses was determined. We found that heat-inactivated VACV cross primed $CD8^+$ T cell responses against peptides from VACV virion antigens. The application of this finding for recombinant vaccines to induce $CD8^+$ T cell responses against foreign antigens was also investigated.

6.2 Results

6.2.1 Heat-inactivated VACV particles prime $CD8^+$ T cell responses against several VACV antigens

(Viruses used in this chapter are listed in Table A-5 in the Appendix)

In order to examine whether heat-inactivated VACV particles induce $CD8^+$ T cell responses in vivo, mice were immunised with VACV strains WR or MVA that were heat-treated at 60°C for 60 min. As shown in Section 3.2.2, this heat inactivation treatment completely inhibits viral gene expression and viral replication. Seven days post immunisation, $CD8^+$ T cell responses were determined in spleens. As seen in Figure 6-1, $CD8^+$ T cell responses specific to several VACV peptides were induced. For mice immunised with heat-inactivated WR, around 0.9% of $CD8^+$ T cells were specific to A3-270, which was equivalent to 8×10^4 A3-270-specific $CD8^+$ T cells (Figure 6-1A and B). The next most dominant were A42-88 and A3-191, in which each was recognised by around

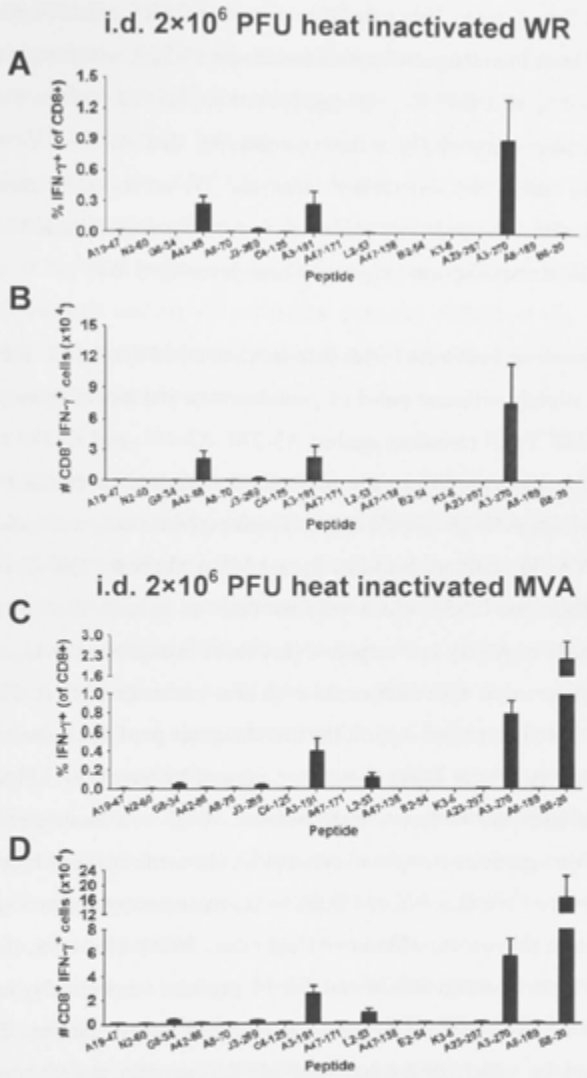


Figure 6-1| CD8$^+$ T cell responses against several VACV peptides can be induced by heat-inactivated VACV virions. VACV strains WR (A and B) and MVA (C and D) were heat-inactivated at 60°C for 60 min and then used for immunise groups of three mice via i.d. injection at a dose of 2×10^6 PFU/mouse, as calculated from the pre-treated value. Seven days post immunisation, peptide-specific CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (A and C) and absolute numbers (B and D) of CD8$^+$ T cells responding to the indicated peptides are shown as mean ± SEM. Results are compiled from two independent experiments (n = 6 for each virus).

0.2% of $CD8^+$ T cells. This translates to around 2×10^4 peptide-specific $CD8^+$ T cells. The least immunogenic peptide tested was J3-289, which was recognised by around 0.05% of $CD8^+$ T cells (equivalent to 2.5×10^3 cells). Some of these responses were surprisingly robust, considering that no VACV antigens were expressed within the immunised animals. However, they remained weak compared with untreated, live WR as shown in Figure 3-1B and C. Importantly, no anti-B8-20 response was induced by heat-inactivated WR.

When mice were immunised with heat-inactivated MVA, $CD8^+$ T cell responses against a slightly different panel of peptides were induced (Figure 6-1C and D). Firstly, $CD8^+$ T cell responses against A3-270, A3-191, and J3-289 were induced, at similar levels as seen in mice immunised with heat-inactivated WR. On the other hand, an A42-88-specific $CD8^+$ T cell response was not induced by heat-killed MVA. In addition, heat-inactivated MVA elicited $CD8^+$ T cell responses against B8-20 and L2-53, which were not detected in mice immunised with heat-inactivated WR. A very low response, just above background level, against G8-34 was also detected in mice immunised with heat-inactivated MVA. Similar to WR, the $CD8^+$ T cell responses against the immunogenic peptides induced by the heat-inactivated MVA were lower than those induced by live MVA (Figure 3-1D and E). It has been shown that a small amount of L2 was associated with mature VACV virion particles using conventional biochemical methods based on western blot analysis of proteins isolated from virion particles and immunogold labelling with electron microscopy (Maruri-Avidal et al., 2011). However, the B8 and G8 antigens, which contain B8-20 and G8-34 peptides respectively, have not been shown to be associated with virions from other VACV strains. Therefore, the mechanism by which heat-inactivated MVA was able to induce $CD8^+$ T cell responses against these two VACV peptides is unclear. These antigens may somehow be trapped within MVA virion particles. No studies have been done to examine which VACV antigens are associated with MVA virions, so the latter hypothesis remains untested.

One possible explanation for the priming of $CD8^+$ T cell responses by the heat-inactivated VACV is that the source of the presented peptides originated from protein impurities present in the virus stock used, but not from the intact VACV

virion particles. The VACV stocks used in this thesis were semi-purified from homogenised VACV-infected cell extracts through sucrose cushion centrifugation. Sucrose cushion centrifugation removes many of the soluble proteins and small debris, but some non-incorporated viral proteins are still present within the prepared VACV stock (Joklik, 1962; Madalinski et al., 1977). The virus stock could also contain other non-antigenic immunoregulatory impurities. It has been suggested that these types of impurities contributes to the immunostimulatory activity of virus-like particles (Deml et al., 2005). It is possible that non-antigenic impurities may have an effect on the $CD8^+$ T cell priming against individual peptides.

To investigate how the purity of VACV stocks influenced the $CD8^+$ T cell immune responses induced by heat-inactivated VACV, two new stocks of WR with different degrees of purity were prepared. A crude stock of WR was prepared by lysing VACV-infected cells through three rounds of freeze-thaw cycles. In addition, a more purified WR stock was prepared through sucrose cushion centrifugation of infected cell extracts, followed by sucrose gradient centrifugation. It has been reported that VACV purified by this process contained minimal level of protein impurities (Joklik, 1962; Zwartouw et al., 1962; Madalinski et al., 1977). Heat-inactivated WR inocula were prepared from these virus preparations, and were injected into mice via the i.d. route. Seven days later, the $CD8^+$ T cells responses against A3-270, A3-191, J3-289 and A42-88 were induced by the heat-inactivated, gradient-purified WR (Figure 6-2). These responses were at similar levels to those detected from mice immunised with heat-inactivated cushion-purified WR (Figure 6-1A and B). One exception was that the heat-inactivated gradient-purified WR seemed to induce a lower anti-A42-88 $CD8^+$ T cell response. Overall, the result here demonstrated that $CD8^+$ T cell responses induced by the inactivated WR were indeed induced by antigens originating from virion particles. When mice were immunised with heat-inactivated crude WR, responses against three additional VACV peptides, namely B8-20, K3-6, and L2-53, were induced, in comparison to the heat-inactivated gradient-purified WR (Figure 6-2). It should be noted that due to variations in responses induced by the heat-inactivated crude WR, the differences in responses against these peptides were not statistically significant when compared with those

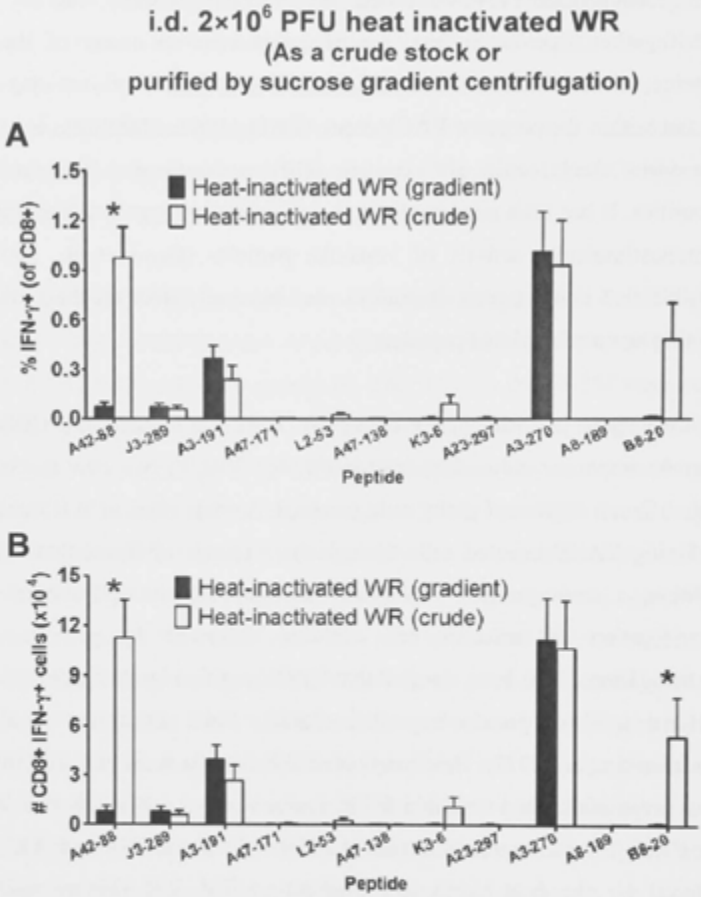


Figure 6-2| CD8$^+$ T cell responses against non-virion proteins can be induced by heat-inactivated WR in a low purity. VACV strain WR prepared as a crude stock (crude) or purified through sequential sucrose cushion and sucrose gradient centrifugation steps (gradient) were heat-treated at 60°C for 60 min. Groups of three mice were immunised i.d. with 2×10^6 PFU of these heat-inactivated viruses. Seven days later, CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Data are shown as mean ± SEM of percentages (A) and absolute numbers (B) of CD8$^+$ T cells responding to the indicated peptides. Results are compiled from two independent experiments (n = 6 for each virus). * denotes statistical significance ($p < 0.05$).

by heat-inactivated gradient-purified WR (Figure 6-2A). However, there was a significant increase in B8-20-specific $CD8^+$ T cell numbers induced by the heat-inactivated crude WR compared to heat-inactivated gradient-purified WR (Figure 6-2B). In addition, the inactivated crude WR induced a significantly higher $CD8^+$ T cell response specific to A42-88 than the inactivated gradient-purified WR (Figure 6-2A and B). These data suggests that impurities within the heat-inactivated inoculum can contribute to $CD8^+$ T cell priming in vivo.

We then investigated whether the induction of a B8-20-specific response by heat-inactivated MVA was due to the presence of MVA B8 antigen within the MVA stock used in Figure 6-1C and D, which was semi-purified by sucrose cushion centrifugation. A new stock of MVA was purified from extracts of MVA-infected cells via sucrose cushion and sucrose gradient centrifugations. The newly prepared gradient-purified MVA as well as the cushion-purified MVA were heat inactivated and were injected into mice. The heat-inactivated gradient-purified MVA induced $CD8^+$ T cell responses against a similar panel of peptides as the heat-inactivated cushion-purified MVA, with some variations in the magnitude of the responses (Figure 6-3). For instance, MVA that was purified via sucrose gradient primed lower $CD8^+$ T cell responses against B8-20, L2-53 and G8-34 than MVA purified via sucrose cushion after heat inactivation, suggesting a role for protein impurities in inducing these responses. Interestingly, a B8-20-specific responses was still elicited by the heat-inactivated gradient-purified MVA, suggesting that the B8 antigen might remain associated with MVA virions after the further purification step. Increasing the purity of the MVA stock also led to an enhancement of responses against A3-270 and J3-289. This effect might be caused by a reduction of competition for MHC-I presentation of peptides sourced from the impurities in the virus stock. Alternatively, the reduction of $CD8^+$ T cell responses against B8-20 might favour priming of $CD8^+$ T cell responses against A3-270 and J3-289. It should be noted that the $CD8^+$ T cell response against A3-191, another $CD8^+$ T cell epitope from the A3 antigen, was not significantly affected by the purity of the virus stock. This suggests that the purity of virus does not influence all the responses equally.

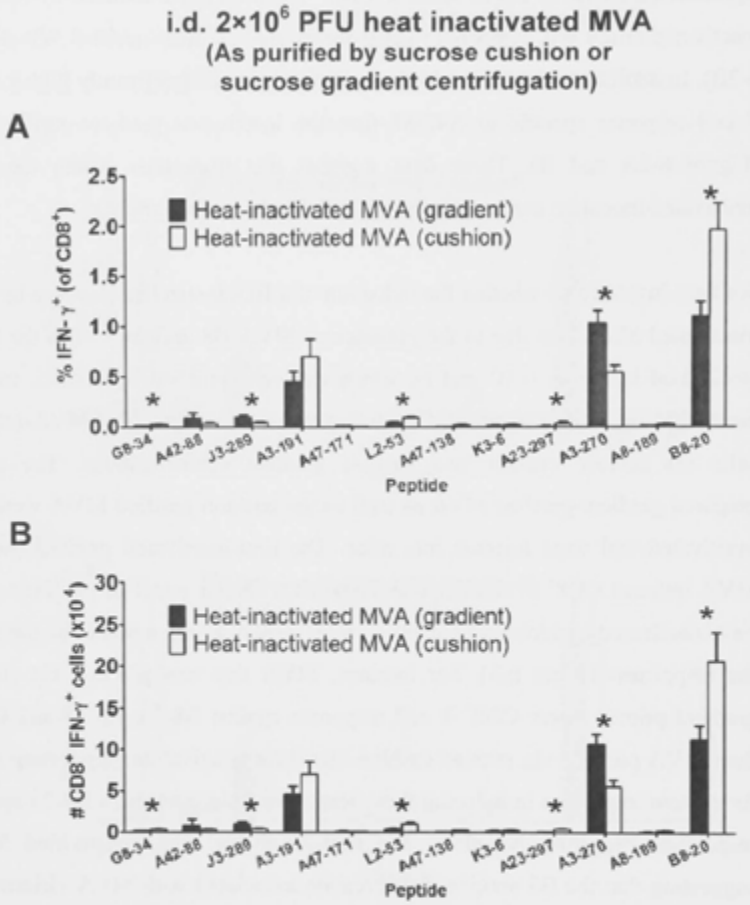


Figure 6-3| A CD8$^+$ T cell response against B8-20 is induced by heat-inactivated gradient-purified MVA virions. MVA purified through a sucrose cushion centrifugation (cushion) or through sequential sucrose cushion and sucrose gradient centrifugation steps (gradient) were heat-treated at 60°C for 60 min. Groups of three mice were then immunised i.d. with the heat-inactivated MVA (2×10^6 PFU/mouse). Seven days post immunisation, peptide-specific CD8$^+$ T cell responses were measured by ex vivo peptide stimulation and ICS and percentages (A) and absolute numbers (B) of CD8$^+$ T cells responding to the indicated peptides were calculated. Results shown are mean ± SEM and are compiled from five independent experiments (n = 15 for each virus). * denotes statistical significance ($p < 0.05$).

6.2.2 The design of a recombinant WR expressing antigenic peptides fused with the A3 core antigen

The results above clearly indicate that $CD8^+$ T cell responses can be primed by heat-inactivated VACV virions. Next, the potential application of heat-inactivated VACV as a recombinant vaccine to induce $CD8^+$ T cell responses against foreign antigens was explored. One criterion for such vaccines to work is that foreign antigens need to be attached to a VACV virion antigen. For this purpose, the A3 core antigen was chosen for the following reasons. Firstly, data in Figure 6-1 shows that the A3 antigen from VACV particles can be used as a substrate for cross priming. Secondly, A3 is relatively abundant within VACV virions (Sarov and Joklik, 1972; Chung et al., 2006). Tagging recombinant antigens to A3 ensures the availability of the foreign antigens on VACV virions for cross priming. Thirdly, WR recombinants expressing A3 fused with fluorescent proteins have been generated (Arakawa et al., 2007; Dodding et al., 2009; Weisswange et al., 2009; Horsington et al., 2012). In all these recombinants, fluorescent proteins are fused to the amino-terminus of A3 and result in fluorescent labelling of the virion particles. These WR recombinants have been used to study how VACV virions are transported within infected cells with fluorescence microscopy (Arakawa et al., 2007; Dodding et al., 2009; Weisswange et al., 2009). Attaching recombinant proteins at the amino-terminus of A3 has been shown not to affect in vitro viral replication and plaque morphology compared to wildtype WR (Arakawa et al., 2007). It should be noted that in wildtype VACV, the first 61 amino acid residues of the amino-terminus of the newly expressed A3 antigen is cleaved to produce the mature core antigen (Moss and Rosenblum, 1973; VanSlyke et al., 1991). However, the successful generation of the fluorescence-labelled VACV particles with fluorescent proteins fused to the amino-terminus of A3 suggests that the removal of the amino-terminal end of the full-length A3 protein may not be necessary.

A recombinant WR, namely WR-eGFP-STS-A3, was generated here. This virus expresses a recombinant antigen, consisting of an eGFP protein, followed by OVA-257, B8-20 and HSV-gB-498 peptides, fused to the amino-terminus of the native full-length A3 antigen (Figure 6-4A). The recombinant antigen tagged to A3 is called eGFP-STS and was designed by Tijana Stefanovic and David

Figure 6-4| The recombinant eGFP-STS-A3 antigen expressed by WR-eGFP-STS-A3. (A) A diagram showing the structure of the recombinant antigen eGFP-STS-A3, consisting of eGFP, OVA-257 peptide, VACV B8-20 peptide and HSV gB-498 peptide tagged onto the amino-terminus of VACV A3 antigen. (B) The amino acid sequence between the end of eGFP and the start of the A3 antigen (position 237 to position 272), is shown. The sequence within the green box represents the last three amino acid residues at the carboxyl-terminus of eGFP. The sequence within the red-, purple- and cyan-coloured boxes represent the sequences of OVA-257, B8-20 and gB-498 peptides respectively. These sequences are separated by a lysine and an alanine residue. The sequence within the blue box corresponds to the first three amino acid residues at the amino-terminus of A3 antigen. (C) The DNA sequence encoding the amino acids in (B) is shown. The sequence in green corresponds to the 3'-end of the sequence encoding eGFP. The DNA sequences highlighted in red, purple and cyan encode the OVA-257, B8-20 and gB-498 peptides respectively. The sequence in blue represents the 5' end of the A3L gene encoding the A3 antigen.

Tscharke (unpublished data). The whole recombinant A3 antigen is called eGFP-STS-A3. The three antigenic peptides on eGFP-STS-A3 are separated by a lysine and an alanine residue (Figure 6-4B and C). These amino acid residues have been shown to allow efficient processing of the flanked peptides from antigens for MHC-I presentation (Eggers et al., 1995; Vijh et al., 1998; Livingston et al., 2001). WR-eGFP-STS-A3 was firstly characterised in vitro. The plaques formed by the recombinant virus on BSC-1 cells after three days of infection were significantly smaller than WR (Figure 6-5A and B). To determine whether this correlated with a growth defect of WR-eGFP-STS-A3, the yield of infectious intracellular virus produced by BSC-1 cells infected with WR or WR-eGFP-STS-A3 at m.o.i of 0.01 was compared. The amount of virus produced by cells infected with WR-eGFP-STS-A3 was significantly lower than WR by at least 2 $\log_{10}$ over the three-day infection period (Figure 6-5C). These results suggest that tagging A3 at the amino-terminus with eGFP-STS antigen results in an attenuation of the recombinant virus in vitro.

The pathogenesis of WR-eGFP-STS-A3 was then examined in vivo. Mice were immunised with live wildtype WR or WR-eGFP-STS-A3 via the i.d. route and the diameter of lesions formed on the infected ear pinna was measured (Tscharke and Smith, 1999). Lesions started to appear on the infected ears at four days post immunisation for both WR and WR-eGFP-STS-A3 (Figure 6-6A). Lesions were significantly smaller for WR-eGFP-STS-A3 than WR from five days post immunisation until the end of experiment seven days after immunisation (Figure 6-6A). After seven days of immunisation, the mice were sacrificed and virus in the infected ears was titrated. The amount of infectious virus found in the ears infected with WR-eGFP-STS-A3 was significantly lower than ears infected with WR. The reduced lesion size and virus growth in the infected ears suggest that WR-eGFP-STS-A3 is less virulent in vivo.

Primary $CD8^+$ T cell responses were also measured in spleens from the immunised mice seven days after immunisation. Only the recombinant virus induced responses against OVA-257 and gB-498 peptides (Figure 6-6C and D), suggesting that the antigenic construct tagged to A3 was immunogenic in vivo. When measured as a percentage of total $CD8^+$ T cells, the anti-B8-20 $CD8^+$ T cell

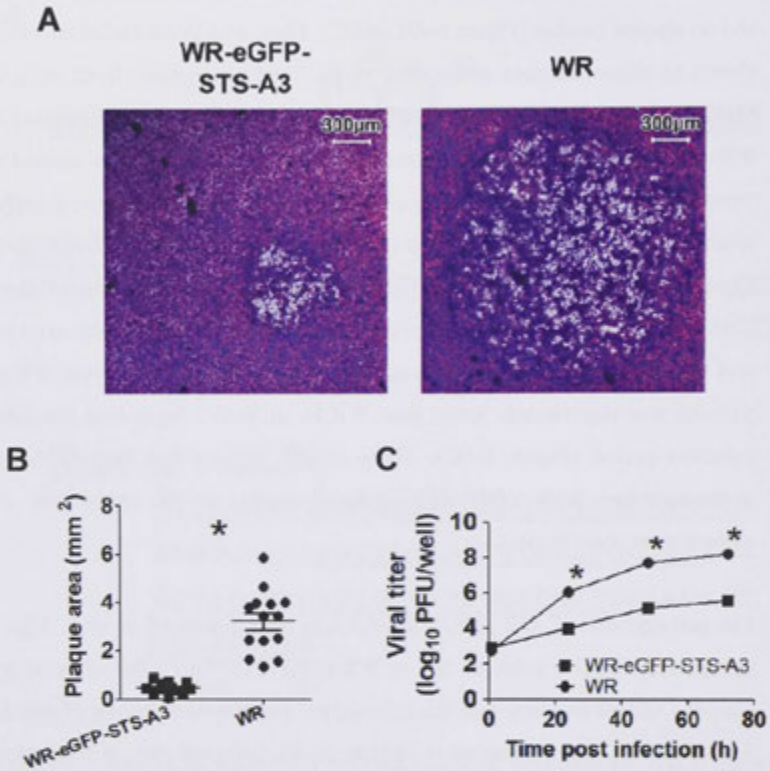


Figure 6-5| WR-eGFP-STS-A3 is attenuated in vitro. (A and B) Confluent BSC-1 cell monolayers in 6-well plates were infected with WR or WR-eGFP-STS-A3 in 10-fold serial dilution for 90 min. Infectious inocula were then replaced with D2 media with 0.4% CMC and incubated at 37°C. Three days later, media were removed and cell monolayers were stained with crystal violet. (A) Representative plaques formed by WR-eGFP-STS-A3 (left) or WR are shown (right). (B) The area of plaques formed by the indicated viruses in cells were measured and are shown as mean ± SEM. (C) the in vitro growth ability of WR-eGFP-STS-A3 was compared with that of wildtype WR. Procedures described in Section 2.2.24.3 were followed. Data are shown as mean ± SEM from triplicate samples (C). (A and B) Data shown are representatives of two similar experiments. (C) Data represent one single experiment. * denotes statistical significance ($p < 0.05$).

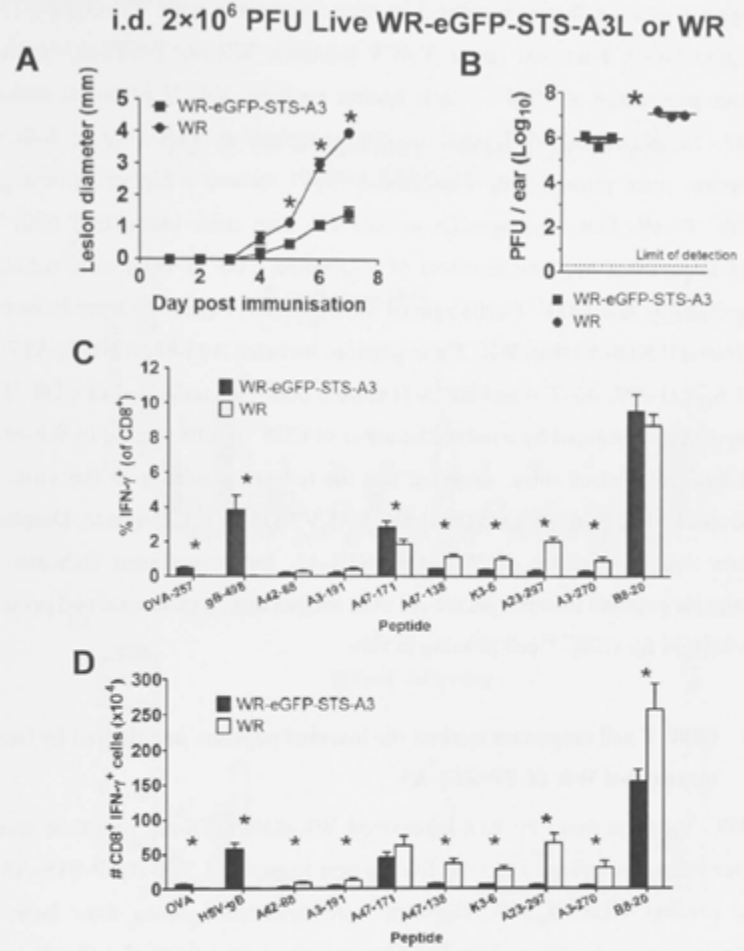


Figure 6-6| WR-eGFP-STS-A3 is attenuated in vivo but can induce CD8$^+$ T cell responses against the inserted antigenic peptides. Groups of three mice were immunised i.d. with 2×10^6 PFU live WR or WR-eGFP-STS-A3. (A) Lesion development was measured on infected ear pinna daily until seven days after immunisation. (B) Seven days after immunisation, infected ears were homogenised and infectious virus levels were determined by standard plaque assay. Virus titres are shown as PFU/ear. (C and D) Seven days post immunisation, CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Percentages (C) and absolute numbers (D) of CD8$^+$ T cells responding to the indicated peptides are shown. All data are shown as mean ± SEM. Data in (A and B) are representatives of two independent experiments while results in (B and C) are compiled from two independent experiments (n = 6 for each virus). * denotes statistical significance ($p < 0.05$).

response was not further increased in mice immunised with WR-eGFP-STS-A3 (Figure 6-6C). For most native VACV peptides, WR-eGFP-STS-A3 primed a lower percentage of $CD8^+$ T cells against multiple VACV peptides, including A47-138, K3-6, A23-297, and A3-270, compared to WR (Figure 6-6C). In contrast, mice primed with WR-eGFP-STS-A3 showed a higher percentage of $CD8^+$ T cells that were specific to A47-171 than mice immunised with WR. However, when the total numbers of responding $CD8^+$ T cells were measured, significantly less $CD8^+$ T cells against multiple VACV peptides were induced by WR-eGFP-STS-A3 than WR. These peptides included A42-88, A3-191, A47-138, K3-6, A23-297, A3-270 and B8-20 (Figure 6-6D). This reduction of $CD8^+$ T cell responses was caused by a reduced number of $CD8^+$ T cells present in WR-eGFP-STS-A3-immunised mice, implying that the reduced virulence of the virus may prevent it from priming a maximal anti-VACV $CD8^+$ T cell response. Despite the lower immunogenicity of WR-eGFP-STS-A3, this experiment indicates that antigenic peptides attached on the A3 core antigen can be processed and presented on MHC-I for $CD8^+$ T cell priming in vivo.

6.2.3 $CD8^+$ T cell responses against the inserted peptides are elicited by heat-inactivated WR-eGFP-STS-A3

$CD8^+$ T cell priming by heat-inactivated WR-eGFP-STS-A3 was then studied. Mice were immunised i.d. with live or heat-inactivated WR-eGFP-STS-A3 and the primary $CD8^+$ T cell responses were determined seven days later. The responses were firstly measured as percentages of total $CD8^+$ T cells (Figure 6-7A). We found that $CD8^+$ T cell responses against OVA-257, gB-498 and B8-20 were induced by heat-inactivated WR-eGFP-STS-A3, demonstrating that antigens tagged to the A3 core antigen can be cross presented from virion particles in vivo for $CD8^+$ T cell priming. The OVA-257-specific response induced by the heat-inactivated virus was significantly higher than that induced by the live virus. A similar $CD8^+$ T cell response against gB-498 was primed by live and heat-inactivated WR-eGFP-STS-A3. A B8-20-specific $CD8^+$ T cell response was also induced by heat-inactivated WR-eGFP-STS-A3, although it was significantly lower than that of the live virus (Figure 6-7A). However because wildtype WR failed to induce a B8-20-specific $CD8^+$ T cell response in vivo after heat

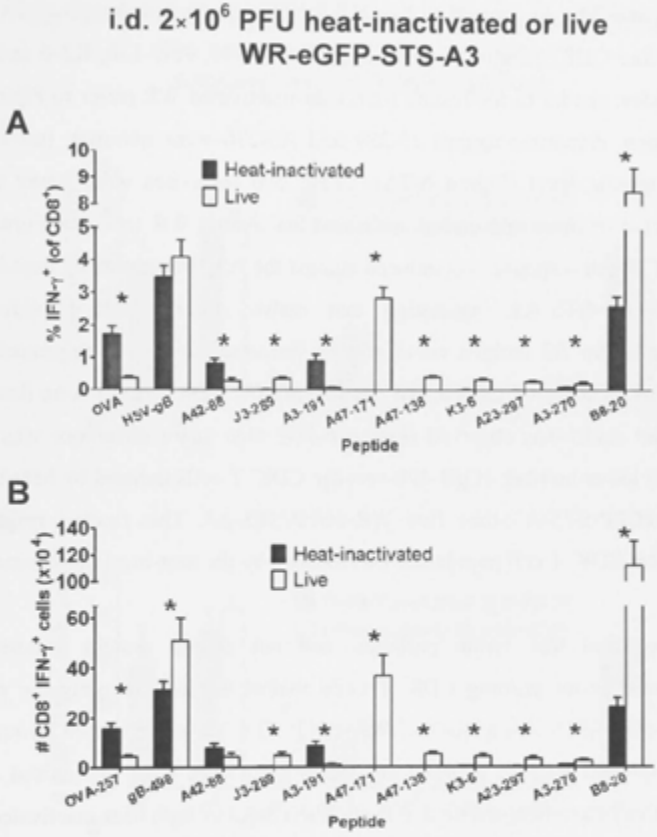


Figure 6-7| Heat-inactivated WR-eGFP-STS-A3 induces $CD8^+$ T cell responses against the inserted antigenic peptides. Groups of three mice were immunised i.d. with live or heat-inactivated WR-eGFP-STS-A3 (2×10^6 PFU/mouse). Seven days later, $CD8^+$ T cell responses in spleens were determined by ex vivo peptide stimulation and ICS. Data are shown as percentages (A) or absolute numbers (B) of $CD8^+$ T cells responding to the peptides indicated. Data are compiled from four independent experiments (n = 12 for each virus). * denotes statistical significance ($p < 0.05$).

inactivation (refer to Figure 6-1), this result suggests that the B8-20-specific $CD8^+$ T cell response was primed by the B8-20 peptide attached to the recombinant A3 antigen. Responses against a panel of native VACV peptides were also measured (Figure 6-7A). In contrast to live eGFP-STS-A3, the heat-inactivated virus failed to prime $CD8^+$ T cell responses against A47-171, A47-138, K3-6 and A23-297 peptides, similar to the results from heat-inactivated WR (refer to Figure 6-1). In addition, responses against J3-289 and A3-270 were minimal, just around the background level (Figure 6-7A). These two responses were lower than those detected in mice immunised with heat-inactivated WR (refer to Figure 6-1). A $CD8^+$ T cell response was induced against the A3-191 peptide by heat-inactivated WR-eGFP-STS-A3, suggesting that native A3 antigenic peptides on the recombinant A3 antigen could still be processed and cross presented from the virions. When total numbers of peptide-specific $CD8^+$ T cells were determined, a similar result was observed (Figure 6-7B). One major difference was that there was a lower number of gB-498-specific $CD8^+$ T cells induced by heat-inactivated WR-eGFP-STS-A3 than live WR-eGFP-STS-A3. This finding reflects that a smaller $CD8^+$ T cell population was induced by the heat-inactivated virus in mice.

To confirm that virion particles, and not simply protein impurities, were responsible for priming $CD8^+$ T cells against the inserted antigenic peptides in WR-eGFP-STS-A3, a stock of WR-eGFP-STS-A3 was prepared through sucrose cushion and sucrose gradient centrifugations. Mice were immunised with heat-inactivated gradient-purified WR-eGFP-STS-A3 or with heat-inactivated cushion-purified WR-eGFP-STS-A3. Seven days post immunisation, similar levels of $CD8^+$ T cell responses against OVA-257, gB-498, and B8-20 were induced by these heat-inactivated virus preparations (Figure 6-8). The gradient-purified virus induced a lower $CD8^+$ T cell response specific to A42-88 which was statistically significant when measured as a total number of A42-88-specific $CD8^+$ T cells (Figure 6-8B). This confirms the previous data with the use of heat-inactivated gradient-purified WR, which also induced a lower A42-88-specific $CD8^+$ T cell response (refer to Figure 6-2). This similarity suggests that the gradient-purified WR-eGFP-STS-A3 indeed contained less impurities than the virus only purified through sucrose cushion centrifugation. Overall results in Figure 6-7 and Figure 6-

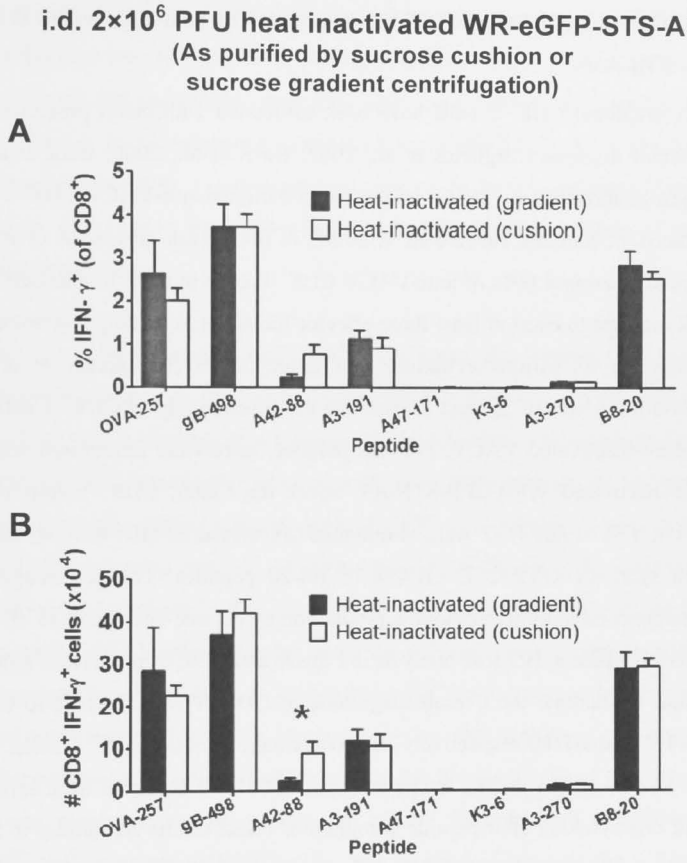


Figure 6-8| Further virus purification does not influence the priming of $CD8^+$ T cell responses against the inserted antigenic peptides by heat-inactivated WR-eGFP-STS-A3. WR-eGFP-STS-A3 purified through sucrose cushion centrifugation (cushion) or further purified with sucrose gradient centrifugation (gradient) was heat-treated at 60°C for 60 min. Groups of three mice were immunised i.d. with 2×10^6 PFU of the heat-inactivated WR-eGFP-STS-A3. Seven days after immunisation, splenic $CD8^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Data are shown as percentages (A) or absolute numbers (B) of $CD8^+$ T cells responding to the peptides indicated. Data are compiled from two independent experiments (n = 6 for each virus). * denotes statistical significance ($p < 0.05$).

8 indicate antigenic peptides tagged to the A3 VACV core antigen can be cross presented to prime $CD8^+$ T cells after complete inactivation of the virus.

6.2.4 The functionality of $CD8^+$ T cells induced by heat-inactivated WR-eGFP-STS-A3

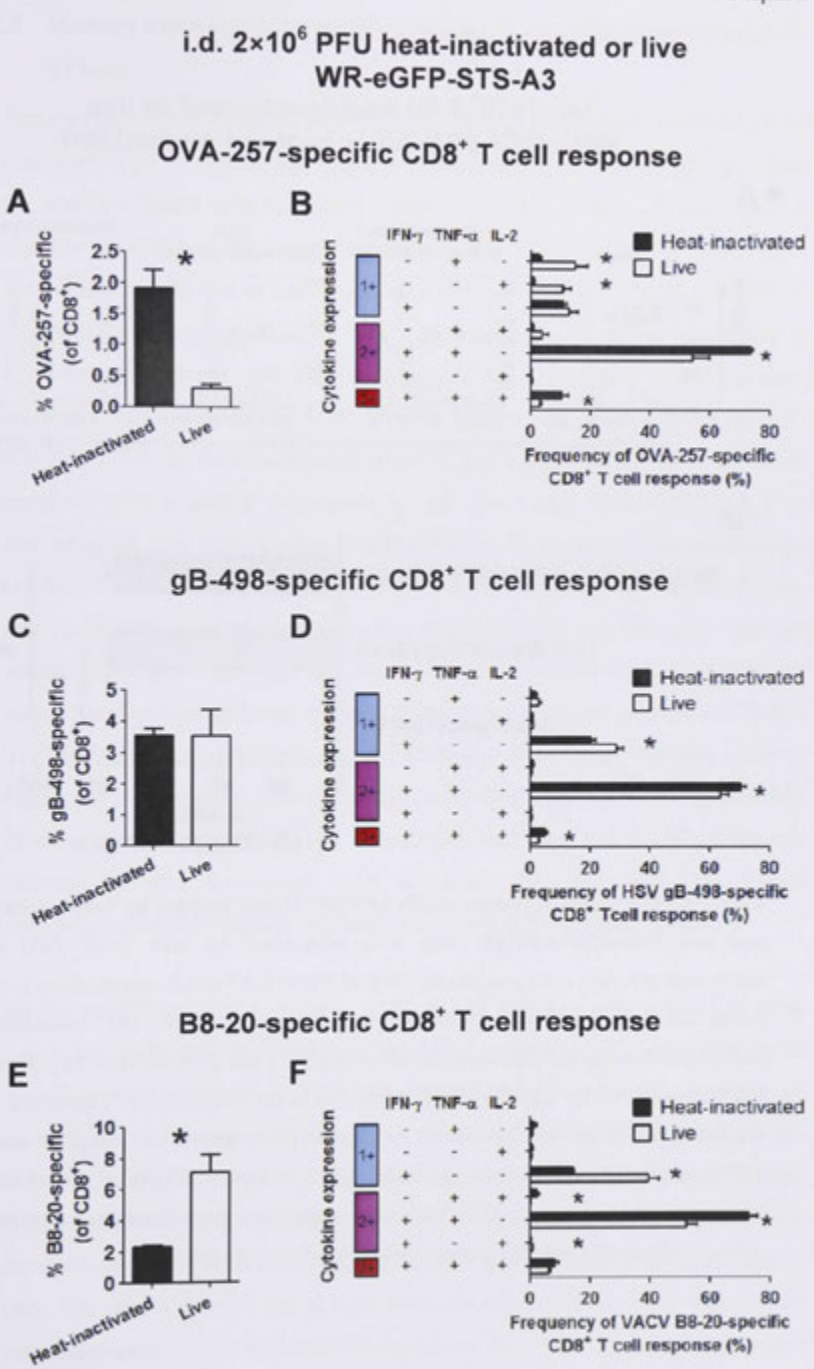
Polyfunctional $CD8^+$ T cells have been associated with better protection against different diseases (Migueles et al., 2002; Betts et al., 2006; Badr et al., 2008; Reyes-Sandoval et al., 2010). Highly polyfunctional anti-VACV $CD8^+$ T cells are induced in humans vaccinated with MVA or Dryvax (Precopio et al., 2007). Similarly, around 60% of anti-VACV $CD8^+$ T cells in mice immunised with live WR are able to exert at least three effector functions, including degranulation and production of various cytokines and granzyme B (Hersperger et al., 2012). Therefore, it was of interest to examine the functionality of $CD8^+$ T cells primed by heat-inactivated VACV. For this purpose, mice were immunised with live or heat-inactivated WR-eGFP-STS-A3. Seven days later, $CD8^+$ T cells producing IFN-γ, TNF-α and IL-2 were simultaneously measured after ex vivo stimulation with synthetic OVA-257, gB-498 or B8-20 peptides. The percentage of each possible combination of cytokine production by peptide-specific $CD8^+$ T cells was determined using Boolean analysis in FlowJo and SPICE softwares. Figures 6-9A, C and E illustrate the overall magnitude of $CD8^+$ T cells specific to OVA-257, gB-498 and B8-20 respectively as a percentage of total $CD8^+$ T cells. The data were calculated by adding the percentages of $CD8^+$ T cells that were expressing at least one cytokine after peptide stimulation. These results are similar to Figure 6-7, where responses are shown as percentages of $CD8^+$ T cell producing IFN-γ. In brief, heat-inactivated WR-eGFP-STS-A3 induced a stronger $CD8^+$ T cell response specific for OVA-257, a similar response to gB-498, and a weaker, but measurable, B8-20-specific response than live WR-eGFP-STS-A3.

In terms of the cytokine expression profile of $CD8^+$ T cells, heat-inactivated eGFP-STS-A3 induced $CD8^+$ T cell responses with similar functionality as the live virus (Figure 6-9B, D and F). The most prominent $CD8^+$ T cell populations against OVA-257, gB-498 and B8-20 induced by either live or heat-inactivated WR-eGFP-STS-A3 were comprised of cells co-expressing IFN-γ and TNF-α.

However, careful analysis showed that there were some variations between the percentages of responding cells expressing individual cytokine combinations. In particular, it seemed that the heat inactivated virus induced larger populations of $CD8^+$ T cells specific to the three examined peptides that co-express IFN-γ and TNF-α than the live virus. In contrast, higher frequencies of $CD8^+$ T cells against OVA-257, gB-498 and B8-20 expressing only one cytokine were induced by live eGFP-STS-A3, in comparison to heat-inactivated eGFP-STS-A3. Overall, these data suggest that $CD8^+$ T cell responses induced by heat-inactivated VACV are similar to, if not better than, those induced by live VACV in terms of multiple cytokine production.

The in vivo cytolytic function of $CD8^+$ T cells was also compared. Seven days after immunisation with live or heat-inactivated WR-eGFP-STS-A3, mice were injected i.v. with a mixture of syngeneic spleen cells that were either pulsed with synthetic gB-498 peptide and labelled with low CFSE intensity or left unpulsed and labelled with a higher concentration of CFSE at a 1:1 ratio. These cells were labelled with the same intensity of Vybrant DiD cell labelling dye, allowing the tracking of the transferred cells. The gB-498-pulsed cells were the targets for gB-498-specific $CD8^+$ T cells while the unpulsed cells acted as an internal control for any non-specific elimination of transferred cells. Four hours after cell transfer, spleens were harvested from the immunised mice, the transferred cell population was determined based on DiD labelling, and the extent of lysis of gB-498-pulsed cells was measured. In naive mice, a similar frequency of gB-498-pulused ($CFSE^{lo}$) and unpulsed ($CFSE^{hi}$) cells was isolated (Figure 6-10A). Similarly, within the spleens of mice immunised with heat-inactivated WR, there was a similar level of the two transferred populations, suggesting that there was a minimal killing of gB-498-pulsed cells in vivo in these mice (Figure 6-10A and B). In mice immunised with either live or heat-inactivated WR-eGFP-STS-A3, however, there was a great loss of gB-498-pulsed cells, but not the unpulsed cells. This translated to around 80% specific killing of gB-498-pulsed cells. There was no statistical difference between mice immunised with heat-inactivated or live WR-eGFP-STS-A3. This experiment indicates that the gB-498-specific $CD8^+$ T cells induced by live and heat-inactivated WR-eGFP-STS-A3 share an equivalently high cytolytic ability in vivo.

Figure 6-9| A comparison of the poly-functionality of $CD8^+$ T cell responses induced by live and heat-inactivated WR-eGFP-STS-A3. Live or heat-inactivated WR-eGFP-STS-A3 were used to immunise groups of three mice (2×10^6 PFU) via the i.d. route. Seven days after immunisation, splenocytes were stimulated ex vivo with synthetic peptides. $CD8^+$ T cells producing IFN-γ, TNF-α and IL-2 were detected with antibody labelling and ICS. The data were then subjected to Boolean analysis with FlowJo software and were further analysed with SPICE software (NIH). Total $CD8^+$ T cell responses against OVA-257 (A), gB-498 (C) and B8-20 (E) were calculated by summing responses of all possible combinations of IFN-γ, TNF-α and IL-2 expression after peptide stimulation. Percentages of $CD8^+$ T cells expressing individual cytokine combinations relative to the total peptide-specific $CD8^+$ T cell responses are shown for OVA-257 (B), gB-498 (D) and B8-20 (F). Data are shown as mean ± SEM and are compiled from two independent experiments (n = 6 for each virus). * denotes statistical significance ($p < 0.05$).


i.d. 2×10^6 PFU heat-inactivated or live WR-eGFP-STS-A3
OVA-257-specific CD8+ T cell response
A
% OVA-257-specific (of CD8+)
2.5
2.0
1.5
1.0
0.5
0.0
*
Heat-inactivated
Live
B
IFN-γ TNF-α IL-2
Cytokine expression
1+
2+
3+
Heat-inactivated
Live
0 20 40 60 80
Frequency of OVA-257-specific CD8+ T cell response (%)
gB-498-specific CD8+ T cell response
C
% gB-498-specific (of CD8+)
5
4
3
2
1
0
Heat-inactivated
Live
D
IFN-γ TNF-α IL-2
Cytokine expression
Heat-inactivated
Live
0 20 40 60 80
Frequency of HSV gB-498-specific CD8+ Tcell response (%)
B8-20-specific CD8+ T cell response
E
% B8-20-specific (of CD8+)
10
8
6
4
2
0
*
Heat-inactivated
Live
F
IFN-γ TNF-α IL-2
Cytokine expression
Heat-inactivated
Live
0 20 40 60 80
Frequency of VACV B8-20-specific CD8+ T cell response (%)

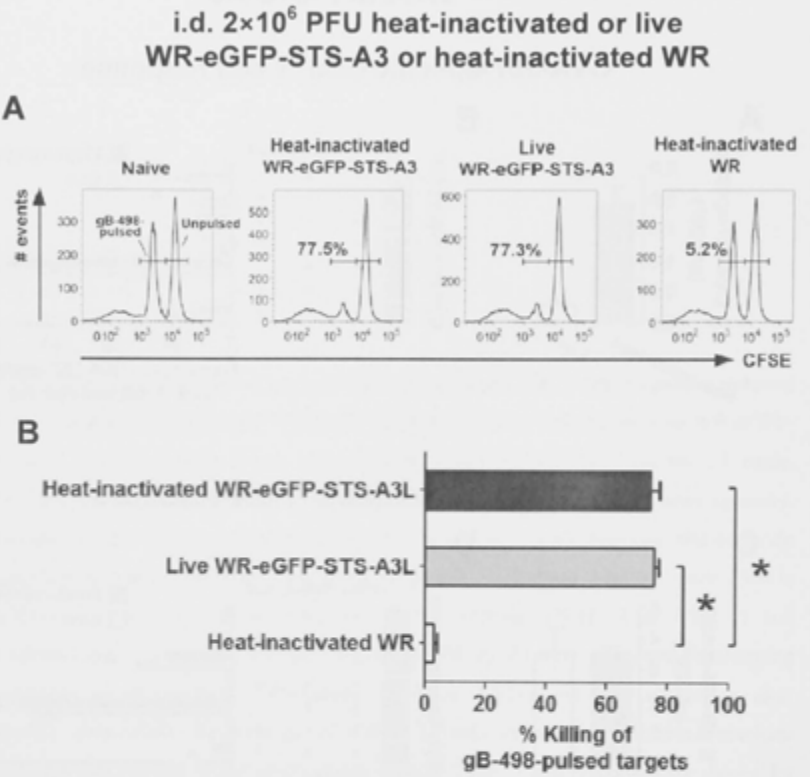


Figure 6-10| A similar cytolytic ability of CD8$^+$ T cells induced by heat-inactivated and live WR-eGFP-STS-A3. Mice were immunised i.d. with 2×10^6 PFU heat-inactivated WR, live or heat-inactivated WR-eGFP-STS-A3 or left unimmunised (naive). Seven days later, in vivo cytotoxicity assay were performed. (A) Representative histograms showing the transferred unpulsed (CFSE^{hi}) and gB-498-pulsed targeted cells (CFSE^{lo}) gated on the transferred cell population in the recipient mice immunised with the indicated viruses. The number in each graph illustrates the percentage of specific killing of gB-498-pulsed targets calculated. (B) The specific killing of gB-498-pulsed targets are shown as mean ± SEM. Data are compiled from two independent experiments with n = 6 for each virus. * denotes statistical significance ($p < 0.05$).

6.2.5 Memory and secondary responses induced by the heat-killed WR-eGFP-STS-A3

Previous data showed that primary $CD8^+$ T cell responses can be induced by heat-inactivated VACV virions. The ability of these $CD8^+$ T cells to be maintained into the memory phase was examined. Memory $CD8^+$ T cell responses against multiple VACV and the inserted peptides were detected at seven weeks after immunisation with live or heat-inactivated WR-eGFP-STS-A3. Memory $CD8^+$ T cells recognised a panel of native VACV peptides that was similar to that seen in the primary response, although as expected the magnitude of the memory responses was lower (Figure 6-11). For the inserted antigenic peptides on WR-eGFP-STS-A3, the heat-inactivated virus induced a gB-498-specific $CD8^+$ T cell response with a similar magnitude as the live virus, when measured as a percentage or as a total number of responding $CD8^+$ T cells. Although the anti-OVA-257 $CD8^+$ T cell response was slightly higher in mice immunised with heat-inactivated WR-eGFP-STS-A3 than live eGFP-STS-A3, the difference was not statistically significant. On the other hand, heat-inactivated eGFP-STS-A3 induced a significantly lower memory $CD8^+$ T cell response against B8-20 than live eGFP-STS-A3, consistent with the finding in the primary response (refer to Figure 6-7). Importantly, heat-inactivated WR failed to induce any detectable B8-20-specific memory $CD8^+$ T cell immunity, confirming that the B8-20-specific response in mice immunised with heat-inactivated WR-eGFP-STS-A3 was induced by the B8-20 peptide originating from the eGFP-STS-A3 construct.

Having established that a memory $CD8^+$ T cell response was induced by heat-inactivated WR-eGFP-STS-A3, we examined whether this could lead to an induction of an enhanced $CD8^+$ T cell response during a heterologous challenge with HSV-1. Mice were immunised i.d. on the left ear with heat-inactivated or live WR-eGFP-STS-A3, live WR-ESmini-gB, live WR or PBS. Seven to eight weeks later, mice were challenged with HSV-1 strain KOS via i.d. injection into the right ear and $CD8^+$ T cell responses against gB-498 were detected seven days later. The results showed that at least 10% of $CD8^+$ T cells in mice primed with heat-inactivated or live WR-eGFP-STS-A3, or live WR-ESmini-gB recognised the gB-498 peptide after HSV-1 infection (Figure 6-12A). In comparison, mice primed with live WR or left unimmunised (mock immunised with PBS) had

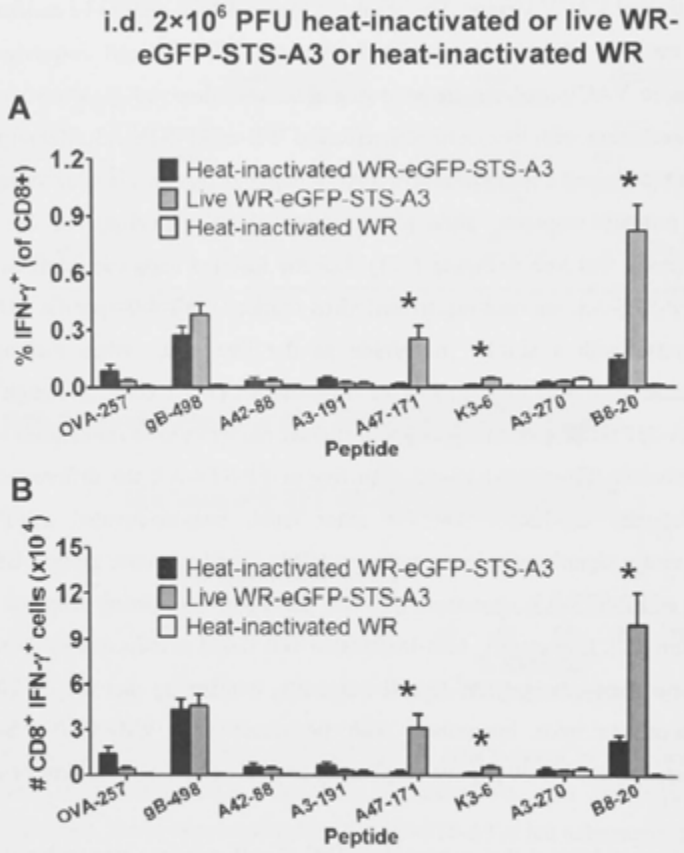


Figure 6-11| Memory CD8$^+$ T cell responses against the inserted antigenic peptides are induced by heat-inactivated WR-eGFP-STS-A3. Groups of three mice were immunised i.d. with 2×10^6 PFU of live or heat-inactivated WR-eGFP-STS-A3. Seven to eight weeks later, CD8$^+$ T cell responses in spleens were determined by ex vivo peptide stimulation and ICS. Percentages (A) or absolute numbers (B) of CD8$^+$ T cells responding to the peptides indicated are shown as mean ± SEM. Data are compiled from three independent experiments (n = 9 for each virus). * denotes statistical significance ($p < 0.05$).

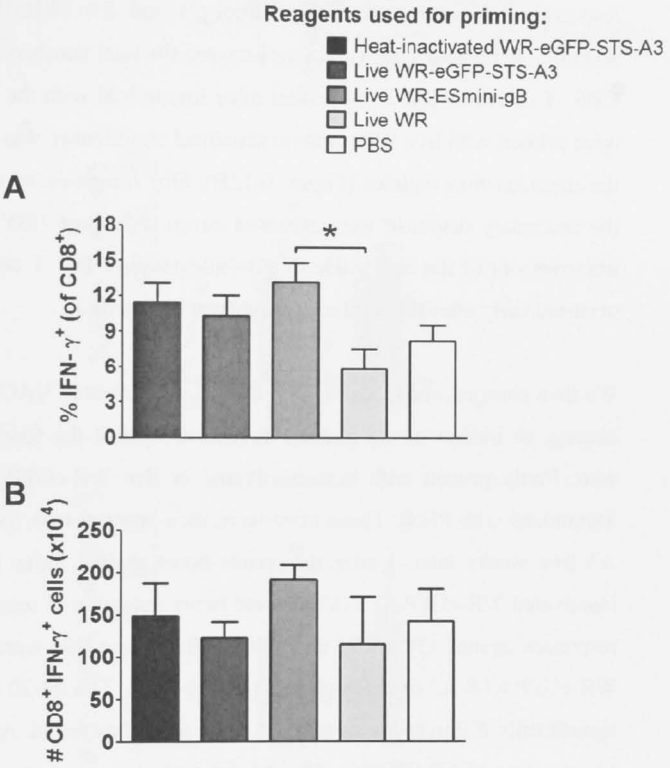


Figure 6-12| Priming with heat-inactivated WR-eGFP-STS-A3 does not enhance the gB-498-specific $CD8^+$ T cell response seven days after HSV-1 challenge. Groups of three mice were injected with PBS or with 2×10^6 PFU of the indicated viruses via i.d. injection into left ear pinna. Seven weeks later, these immunised mice were infected with 5×10^5 PFU HSV-1 by i.d. injection into right ear pinna. Seven days after HSV-I challenge, gB-498-specific $CD8^+$ T cell responses in spleens were determined by ex vivo peptide stimulation and ICS. Percentages (A) or absolute numbers (B) are shown as mean ± SEM. Data are compiled from two independent experiments (n = 6 for each virus). * denotes statistical significance ($p < 0.05$).

around or less than 8% of $CD8^+$ T cells specific to gB-498 after the heterologous challenge. However, the only statistical difference detected was between the responses induced by live WR-ESmini-gB and live WR (Figure 6-12A). In addition, there were great variations between the total numbers of gB-498-specific $CD8^+$ T cells induced in individual mice immunised with the same reagent (e.g. mice primed with live WR), and no statistical significance was detected across all the immunisation regimes (Figure 6-12B). One limitation of this assay was that the secondary response was measured seven days post HSV-1 challenge. Any improvement of the magnitude of gB-498-specific $CD8^+$ T cell responses which occurred early after the challenge would not be detected.

We then changed our focus to the use of heat-inactivated VACV in a prime-boost strategy to induce strong immune responses against the foreign antigens. Mice were firstly primed with heat-inactivated or live WR-eGFP-STS-A3 (or mock immunised with PBS). These mice were then boosted with live WR-eGFP-STS-A3 five weeks later. Under this prime-boost regime, mice primed with heat-inactivated WR-eGFP-STS-A3 allowed better induction of secondary $CD8^+$ T cell responses against OVA-257 and gB-498 than mice that were primed with live WR-eGFP-STS-A3 or mock-primed (Figure 6-13). The B8-20 responses were not significantly different across all the priming reagents tested. As seen in Figure 6-11, the live eGFP-STS-A3 induced a relatively strong memory $CD8^+$ T cell responses against B8-20 compared to heat-inactivated virus. This large B8-20-specific memory population might interfere with the successive induction of secondary responses against other antigenic peptides. Furthermore, the antibody level induced by the live virus might be substantially higher than that induced by the heat-inactivated virus during the priming phase (Madeley, 1968; Turner et al., 1970). This might suppress the ability of WR-eGFP-STS-A3 to induce secondary responses as a homologous boosting vaccine. In summary, these data show that priming with heat-inactivated virus and boosting with live virus is superior to a live-prime-live-boost strategy.

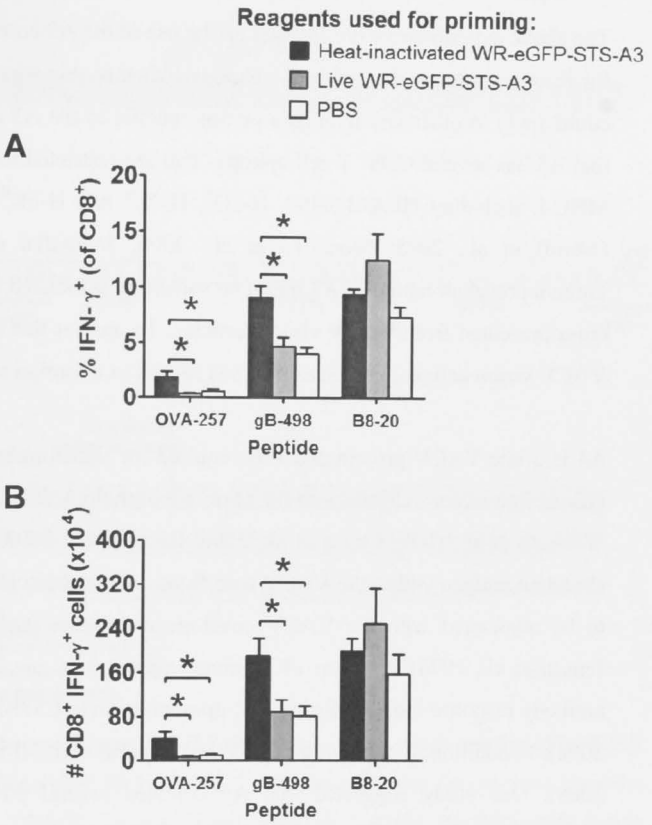


Figure 6-13| Priming with heat-inactivated WR-eGFP--STS-A3 improves the secondary CD8$^+$ T cell responses against the inserted antigenic peptides after boosting with live WR-eGFP-STS-A3. Groups of three mice were injected with PBS or with 2×10^6 PFU of live or heat-inactivated WR-EGFP-STS-A3 via i.d. injection into left ear pinnae. Five weeks later, the immunised mice were boosted with 2×10^6 PFU live WR-eGFP-STS-A3 by i.d. injection into right ear pinnae. Seven days later, peptide-specific CD8$^+$ T cell responses in spleens were determined by ex vivo peptide stimulation and ICS and shown as percentages (A) or absolute numbers (B) of CD8$^+$ T cells responding to the peptides indicated. Data are shown as mean ± SEM and are compiled from three independent experiments with n = 9 for each experimental group. * denotes statistical significance ($p < 0.05$).

6.2.6 The design of VACV recombinants expressing foreign antigenic peptides tagged to the A4 core antigen

The above experiments were focused on the use of the A3 core antigen as a carrier for foreign antigens. It remained unknown whether this was a general role that could apply to other virion antigen or was specific to the A3 antigen. It is known that A3 has several $CD8^+$ T cell epitopes that are restricted to human and murine MHC-I, including HLA-B*4403, H-$2D^b$, H-$2K^b$ and H-$2K^d$ (Jing et al., 2005; Oseroff et al., 2005; Moutaftsi et al., 2006; Moutaftsi et al., 2010). This immunoprevalent nature of A3 might be somehow associated with its ability to be cross presented from VACV virion particles. To answer this question, a different VACV virion antigen, known as A4, was chosen as a foreign antigen carrier.

A4 is a late VACV protein and it is required for transforming immature VACV virions into mature virions with the correct morphology (Maa and Esteban, 1987; Williams et al., 1999; Chung et al., 2006; Resch et al., 2007). This antigen is an abundant antigen within the VACV core (Maa and Esteban, 1987). It is also found to be associated with the VACV envelope membrane (Cudmore et al., 1996; Jensen et al., 1996). In terms of immunogenicity, it is one of the targets of the antibody response induced during WR immunisation in C57Bl/6 mice (Sette et al., 2008). In addition, it contains a peptide restricted to MHC-II H-2-IA^b (Sette et al., 2008). One study suggested that A4 contains several putative $CD8^+$ T cell epitopes, in which their responses could be induced by immunisation with DNA encoding the A4 antigen (Otero et al., 2006). However, in contrast to A3, no $CD8^+$ T cell epitopes have been defined in the context of VACV immunisation of mice or humans. Similar to the A3 antigen, the A4 antigen has also been used as a fusion target for fluorescent proteins to generate fluorescently-labelled VACV particles (Carter et al., 2003; Carter et al., 2005; Herrero-Martínez et al., 2005; Huang et al., 2008; Mercer and Helenius, 2008).

For our study, a slightly different antigenic construct used for tagging A3 was used here. The construct was called eGFP-SKS, which consists of a eGFP protein, followed by OVA-257, D3E-128 and gB-498 peptides (Figure 6-14). The D3E-128 peptide is a newly mapped $CD8^+$ T cell epitope at position 128-135 of dengue

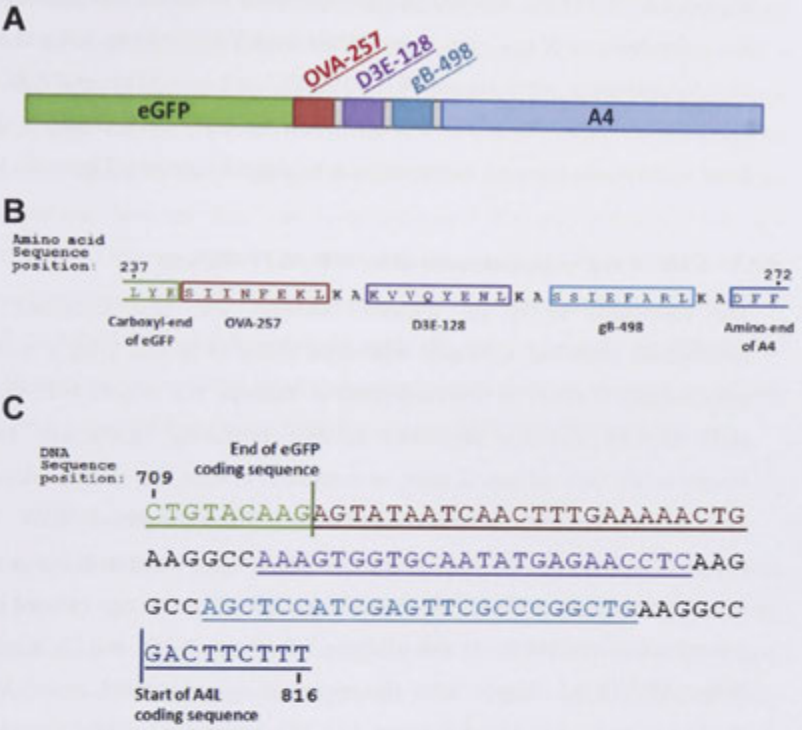


Figure 6-14| The recombinant eGFP-SKS-A4 antigen expressed by WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4. (A) A schematic showing the antigenic construct consisting eGFP, OVA-257 peptide, D3E-128 peptide and HSV gB-498 peptide tagged onto the amino-terminus of VACV A4 antigen, creating the recombinant eGFP-SKS-A4 protein. (B) The amino acid sequence between the end of eGFP and the start of A4 antigen, corresponding to position 237 to position 272 of the recombinant eGFP-SKS-A4 antigen, is shown. The sequence highlighted within the green box represents the last three amino acid residues at the carboxyl-terminus of eGFP. The sequence within the red-, purple- and cyan-coloured boxes represent OVA-257, D3E-128 and gB-498 peptide sequences respectively. The sequence within the blue box corresponds to the first three amino acid residues at the amino-terminus of A4 antigen. (C) The DNA sequence encoding the amino acids illustrated in (B) is shown. The sequence in green corresponds to the 3'-end of the sequence encoding eGFP. The DNA sequences highlighted in red, purple and cyan encode the OVA-257, D3E-128 and gB-498 peptides respectively. The sequence in blue represents the 5' end of the A4L gene encoding the A4 antigen.

virus type 3 envelope protein that is predicted to bind to H-2K^b (amino acid sequence KVVQYENL; Bárbara Quinan and David Tscharke, unpublished data). We generated a set of two virus recombinants from VACV strains WR and MVA. The recombinants were named WR-eGFP-SKS-A4 and MVA-eGFP-SKS-A4 respectively. These two recombinants express the eGFP-SKS construct that is fused to the amino-terminus of the native full-length A4 antigen (Figure 6-14).

6.2.7 CD8$^+$ T cell responses induced by WR-eGFP-SKS-A4

The replication ability of WR-eGFP-SKS-A4 was first examined. This recombinant virus had a reduced replication ability of around 2 $\log_{10}$ in BSC-1 cells infected at m.o.i. of 0.01, compared to wildtype WR (Figure 6-15A). WR-eGFP-SKS-A4 was also attenuated in vivo, producing significantly smaller lesions on the infected ears in mice, as measured between four to seven days post immunisation, compared to WR (Figure 6-15B). When the primary CD8$^+$ T cell responses induced by WR and WR-eGFP-SKS-A4 were compared, it was found that responses against OVA-257, D3E-128 and gB-498 were only induced by the recombinant virus, but not by the wildtype WR (Figure 6-15C and D). Similar to WR-eGFP-STS-A3 (Figure 6-6), the responses against several native VACV peptides, namely A47-138, K3-6, and A23-297, induced by eGFP-SKS-A4 were significantly less than those of WR when the responses were measured as percentages of total CD8$^+$ T cells (Figure 6-15C). Because a smaller population of CD8$^+$ T cells were present in mice immunised with WR-eGFP-SKS-A4 than WR, the total number of CD8$^+$ T cells specific to A47-138, K3-6, A23-297, A3-270 and B8-20 were also decreased (Figure 6-15D). Overall, eGFP-SKS-A4 seemed to behave similarly to WR-eGFP-STS-A3: both are attenuated and induce a less potent anti-VACV response.

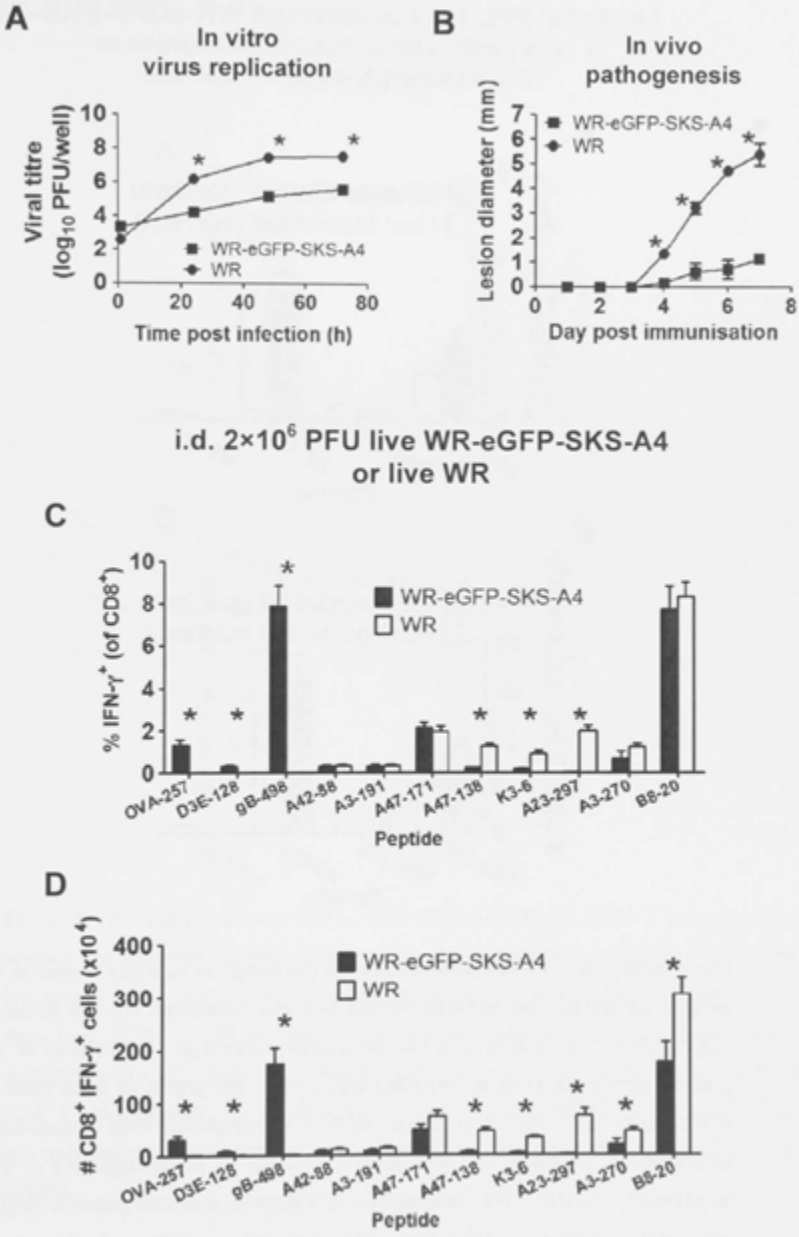
The immunogenicity of heat-inactivated WR-eGFP-SKS-A4 was then investigated. When mice were immunised with heat-inactivated WR-eGFP-SKS-A4 purified though sucrose cushion centrifugation, responses against OVA-257, D3E-128, and gB-498 were induced (Figure 6-16). A very low level CD8$^+$ T cell response against B8-20 was also primed by the heat-inactivated virus, unlike previous findings with heat-inactivated WR (Figure 6-1A to C). However, this

minute B8-20-specific response was not detected in mice immunised with heat-inactivated WR-eGFP-SKS-A4 that was purified via sucrose cushion and sucrose gradient centrifugations (Figure 6-16). This suggests that B8 antigen was a contaminant in the cushion-purified virus stock. In comparison, further virus purification did not influence the $CD8^+$ T cell immunity against the antigenic peptides tagged on the A4 antigen primed by the heat-inactivated virus. A direct comparison between live and heat-inactivated WR-eGFP-SKS-A4 was not performed. However, when compared with the results on Figure 6-15, live and heat-inactivated WR-EGFP-SKS-A4 appeared to induce similar levels of OVA-257- and D3E-128-specific responses while the response against gB-498 seemed to be smaller. Overall, this result demonstrates that antigenic peptides on A4 can be cross presented from virion particles to prime $CD8^+$ T cell responses.

6.2.8 $CD8^+$ T cell responses induced by MVA-eGFP-SKS-A4

To examine whether recombinant MVA could also be used as a heat-inactivated vaccine to induce $CD8^+$ T cell responses against foreign antigens, the immunogenicity of live and heat-inactivated MVA-eGFP-SKS-A4 was compared. Live MVA-eGFP-SKS-A4 elicited primary $CD8^+$ T cell responses against OVA-257, D3E-128 and gB-498, the three antigenic peptides on the recombinant eGFP-SKS-A4 antigen, as measured seven days post immunisation (Figure 16-17). This experiment shows that antigenic peptides available on A4 were processed for $CD8^+$ T cell priming when expressed from MVA. When mice were immunised with heat-inactivated MVA-eGFP-SKS-A4, these three foreign antigenic peptides remained immunogenic. However, the magnitude of the induced responses against OVA-257 and gB-498 were significantly lower than those induced by the live virus. As expected, the anti-B8-20 response was lower in mice immunised with heat-inactivated MVA-eGFP-SKS-A4, compared to live MVA-eGFP-SKS-A4. The data here suggests that antigenic peptides tagged on A4 can be cross presented from heat-inactivated MVA virion particles to prime CD^+ T cell responses although it is less immunogenic that the live MVA recombinant.

Figure 6-15| WR-eGFP-SKS-A4 is attenuated in vitro and in vivo. (A) In vitro growth ability of WR-eGFP-SKS-A4 was compared to that of wildtype WR. Procedures described in Section 2.2.24.3 were followed. Results are shown as mean ± SEM from triplicate samples. (B, C and D) Groups of three mice were immunised i.d. with 2×10^6 PFU of live WR-eGFP-SKS-A4 or live WR. (B) Diameter of lesions was measured on infected ear pinna daily for seven days. (C and D) Seven days post immunisation, $CD8^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. The results are shown as mean ± SEM of percentages (C) and absolute numbers (D) of $CD8^+$ T cells responding to the indicated peptides. Data shown in (A) are from one single experiment. Data in (B) are representative of two independent experiments (n = 3 for each virus) while results in (C and D) are compiled from two independent experiments (n = 6 for each virus). * denotes statistical significance ($p < 0.05$).


A
In vitro
virus replication
Viral titre
(log10 PFU/well)
WR-eGFP-SKS-A4
WR
Time post infection (h)
B
In vivo
pathogenesis
Lesion diameter (mm)
WR-eGFP-SKS-A4
WR
Day post immunisation
i.d. 2×10^6 PFU live WR-eGFP-SKS-A4
or live WR
C
% IFN-γ+ (of CD8+)
WR-eGFP-SKS-A4
WR
OVA-257
D3E-128
gB-498
A42-88
A3-191
A47-171
A47-138
K3-6
A23-297
A3-270
B8-20
Peptide
D
CD8+ IFN-γ+ cells (x10^4)
WR-eGFP-SKS-A4
WR
OVA-257
D3E-128
gB-498
A42-88
A3-191
A47-171
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K3-6
A23-297
A3-270
B8-20
Peptide

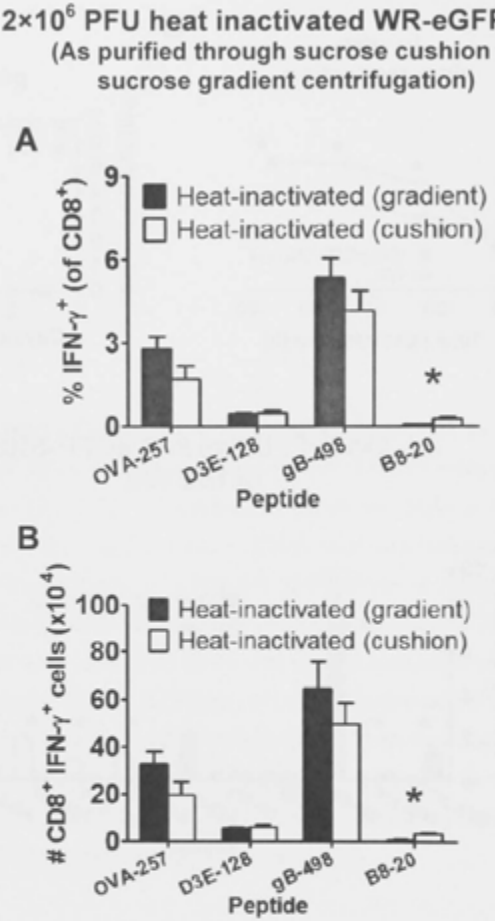

Figure 6-16| CD8$^+$ T cell responses against the antigenic peptides tagged to the A4 antigen are induced by heat-inactivated WR-eGFP-SKS-A4. Heat-inactivated WR-eGFP-SKS-A4 purified through sucrose cushion centrifugation (cushion) or sucrose gradient centrifugation (gradient) were injected i.d. into groups of three mice (2×10^6 PFU/mouse). Seven days later, splenic CD8$^+$ T cell responses were determined by ex vivo peptide stimulation and ICS. Data are shown as percentages (A) or absolute numbers (B) of CD8$^+$ T cells responding to the peptides indicated (mean ± SEM). Data are compiled from two independent experiments with n = 6 for each virus. * denotes statistical significance ($p < 0.05$).

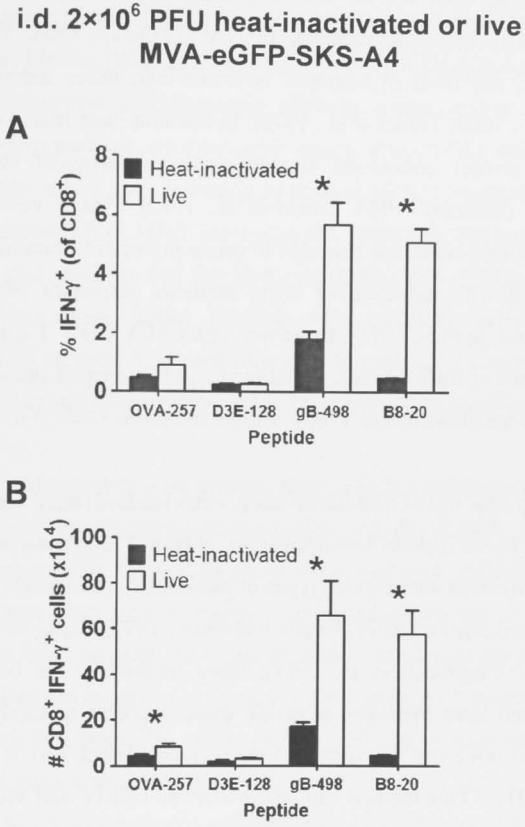

Figure 6-17| Heat-inactivated MVA-eGFP-SKS-A4 induces $CD8^+$ T cell responses against the antigenic peptides tagged to the A4 antigen. Groups of three mice were immunised i.d. with 2×10^6 PFU live or heat-inactivated MVA-eGFP-SKS-A4. Seven days later, $CD8^+$ T cell responses in spleens were determined by ex vivo peptide stimulation and ICS. Data are shown as percentages (A) or absolute numbers (B) of $CD8^+$ T cells responding to the peptides indicated (mean ± SEM) and are compiled from two independent experiments (n = 6 for each virus). * denotes statistical significance ($p < 0.05$).

6.3 Discussion

It has long been known that heat-inactivated VACV can induce antibody responses in immunised animals in vivo (Madeley, 1968; Turner et al., 1970). However, the level of response is lower than those induced by live VACV (Madeley, 1968; Turner et al., 1970). In addition, heat-inactivated VACV are less able to protect immunised animals against subsequent challenges with live poxvirus (Madeley, 1968, Turner et al., 1970). VACV vaccines inactivated by other methods were also less able to prime protective immunity in vivo and were associated with induction of lower antibody responses (Boulter et al., 1971, Turner and Squires, 1971). However, anti-VACV $CD8^+$ T cell immunity was not determined in these studies. Experiments in this chapter demonstrate for the first time that heat-inactivated VACV virions can prime $CD8^+$ T cells in vivo.

Other than the use of heat inactivation, other methods have been used to inactivate VACV. These include UV irradiation, chemical inactivation with formaldehyde and inactivation with various types of photoactive chemicals (Boulter et al., 1971; Turner and Squires, 1971; Hearst and Thiry, 1977; Tsung et al., 1996; Fischer et al., 2007; Sagripanti et al., 2011). More recently, it has been shown that WR inactivated with hydrogen peroxide induces potent antibody responses against VACV in mice and provides protection against lethal VACV challenge (Amanna et al., 2012). This method can also inactivate LCMV and West Nile virus which can be used to induce a $CD8^+$ T cell response in vivo (Amanna et al., 2012; Walker et al., 2012; Pinto et al., 2013). These studies suggest that viral antigens on hydrogen peroxide-inactivated virion particles can be cross presented for $CD8^+$ T cell priming. However, it has yet to be confirmed that hydrogen peroxide can completely abolish viral gene expression. In contrast, data shown in Section 3.2.2 illustrated that heat-inactivation completely inhibits VACV viral replication and gene expression.

Results shown in this chapter clearly demonstrate that VACV antigens from heat-inactivated virion particles can be used as the substrates for cross priming $CD8^+$ T cells in vivo. This mode of cross priming has not been previously reported for VACV. Furthermore, $CD8^+$ T cell responses could also be induced by protein

impurities within the heat-inactivated VACV that was used for immunisation. However, it remains puzzling how B8-20-specific $CD8^+$ T cells were primed by heat-inactivated gradient-purified MVA. The B8 antigen expressed from MVA is truncated compared to the B8 antigen expressed from WR (Antoine et al., 1998). One possibility is that truncated B8 protein might be trapped within MVA virion particles during virus packing. An alternative reason is that MVA virion particles may be more sticky and it may be harder to remove protein impurities from the MVA stocks. A recombinant MVA expressing the full-length B8 antigen of WR could be made and used to test the first possibility. For testing the second possibility, a MVA stock further purified using caesium chloride gradient centrifugation could be used. This method is sensitive enough to separate various types of VACV virion particles (Planterose et al., 1962; Payne and Norrby, 1976).

Another unexamined aspect of cross priming from VACV particles is whether this mechanism happens naturally during immunisation with live VACV. It is difficult to separate in vivo priming induced by newly-expressed viral antigens and those induced by antigens from virus particles. One possible strategy is to create a recombinant virus that expresses the virion antigen of interest under an inducible promoter. This virus can be grown in vitro with the presence of the inducer. After immunisation with this virus, the virion antigen will not be expressed in vivo. This recombinant virus is probably unable to replicate in vivo and so it is as best to compare with MVA which cannot replicate. However, this recombinant would not mimic a situation where the virus is replicated in vivo, as for WR. It is possible that viral particles generated in vivo can be used as substrate for cross priming. On the other hand, perhaps heat inactivation structurally modifies VACV virions in a way that promotes cross presentation in vivo. A different inactivation method, such as irradiation of VACV with germicidal UV (i.e. UV in 254 nm wavelength; Norbury et al., 2004; Lev et al., 2009), could be used to examine whether inactivation method influences cross presentation of antigens from VACV particles.

This chapter also suggests that recombinant antigens fused with VACV core proteins can be incorporated into VACV virions and can be processed from inactivated VACV particles for $CD8^+$ T cell priming in vivo. However, several

questions remain to be answered. Firstly the uptake mechanism of heat-inactivated VACV has not been examined. It has been shown that the live VACV particle can be taken up by cells, including DCs, via macropinocytosis into an intracellular vesicle (Mercer and Helenius, 2008; Mercer et al., 2010; Sandgren et al., 2010). The VACV core is then released into the cytoplasm via fusion of the VACV envelope membrane with the vesicle membrane, which requires interactions between multiple VACV and cellular proteins (Laliberte et al., 2011). The internalisation process of VACV mature virions via macropinocytosis requires the presence of phosphatidylserine or other similar anionic phospholipids on the viral envelope membrane (Ichihashi and Oie, 1983; Mercer and Helenius, 2008; Mercer et al., 2010). Phosphatidylserine is heat stable (Ichihashi and Oie, 1983). Therefore, this lipid may still be available on heat-inactivated VACV to trigger macropinocytosis, so long as the viral envelope membrane remains intact after heat treatment. However, because heat denatures proteins, the release of the heat-inactivated VACV core into the cytoplasm might not be allowed. It has been shown that HIV particles taken up by macrophages via macropinocytosis are degraded within the vesicles (Maréchal et al., 2001; Gobeil et al., 2013). Heat-inactivated VACV internalised by DCs might be partially degraded to release viral antigens (either as intact or partially processed forms). These virion antigens might then be further processed by the possible processing pathways discussed in Section 1.7.2 for cross presentation.

Alternatively, heat-inactivated VACV may be taken up by APCs via a completely different pathway from live VACV. Two recent reports have identified actin filament (formed from polymerisation of actin subunits) as the ligand for the Clec9A receptor (Ahrens et al., 2012; Zhang et al., 2012), an important receptor on DCs for sensing dead cells and for promoting cross presentation of antigens associated with dead cells (Sancho et al., 2009). This receptor plays a role in directing captured dead-cell-associated antigens into endosomes, which favour cross presentation onto MHC-I, instead of lysosomes that would lead to extensive antigen degradation (Sancho et al., 2009; Zelenay et al., 2012). Targeting recombinant antigens to Clec9A by conjugating with anti-clec9A antibody promotes enodocytosis and cross presentation of the antigens (Caminschi et al., 2008; Sancho et al., 2008; Lahoud et al., 2011). Interestingly, actin subunits have

been identified on VACV virion particles (Jensen et al., 1996; Chung et al., 2006; Resch et al., 2007). If the virion-associated actin is in the form of filaments, it is possible that heat-inactivated VACV binds to Clec-9A directly and promotes cross presentation. Finally, heat-inactivated viruses may form large antigenic aggregates which are captured by DCs.

We have established an in vitro cross presentation assay for detecting antigen presentation on cells co-cultured with heat-inactivated VACV. In this assay, BMDCs are co-cultured with heat-inactivated WR-eGFP-STS-A3 and are then used as stimulators to drive the proliferation of CFSE-labelled naive $CD8^+$ T cells isolated from OT-I mice. Using this assay, OVA-257 presentation was detected on cells co-cultured with heat-inactivated WR-eGFP-STS-A3, but not with heat inactivated WR-mini-OVA, which expresses the cytosolic OVA-257 minigene (data not shown). Together with inhibitors to stop specific antigen processing pathways or with BMDCs generated from mice deficient in certain antigen presentation pathways, this assay would allow in vitro dissection of how antigens from heat-inactivated VACV virions are captured and processed for cross presentation.

How antigen-presenting DCs are activated by heat-inactivated VACV was not examined in this chapter. Several publications have shown that UV-inactivated MVA and WR can activate co-cultured DCs or macrophages in vitro to produce inflammatory cytokines (Zhu et al., 2007; Delaloye et al., 2009; Waibler et al., 2009). In addition, VACV strains Copenhagen and MVA inactivated by UV or heat (at 55°C) induced expression of the costimulatory molecule CD86 on co-cultured DCs (Drillien et al., 2004). Several TLR might be involved (Zhu et al., 2007; Samuelsson et al., 2008; Delaloye et al., 2009; Bauer et al., 2010; Martinez et al., 2010a) but their precise roles remain controversial (Cervantes et al., 2012). More recently, Ferguson et al. (2012) found that DNA-dependent protein kinase can bind to transfected DNA isolated from VACV to activate innate immune responses pathway in fibroblasts in vitro. The investigators further demonstrated that DNA-dependent protein kinase contributes to IL-6 and IFN-β production in the infected ears after MVA immunisation via i.d. injection in vivo (Ferguson et al., 2012). However, its role for heat-inactivated VACV remains unknown.

Overall, the mechanism of DC activation by heat-inactivated VACV should be investigated. The use of BMDCs generated from mice that are TLR-deficient could be a start. However, DCs involved in $CD8^+$ T cell priming in vivo during immunisation with heat VACV should first be identified as they might require a different activation pathway compared with the in vitro-generated APCs.

The recombinant VACV generated in this chapter expressed foreign antigens that are fused to either the A3 or A4 VACV core antigen. It remains unclear whether VACV envelope antigens can be used as antigen carriers in heat-inactivated VACV. None of the native VACV peptides tested in this thesis originated from envelope membrane proteins. Peptides from such proteins have been reported as $CD8^+$ T cell epitopes in mice or humans immunised with live VACV although they are under-represented (Oseroff et al., 2005; Pasquetto et al., 2005; Moutaftsi et al., 2006; Tang et al., 2006; Oseroff et al., 2008). Whether these proteins are also immunogenic after VACV inactivation needs to be examined and this may provide more possible VACV antigens for fusing with recombinant antigens.

There are several difficulties which need to be tackled to improve heat-inactivated VACV as recombinant vaccines. Firstly, as seen for WR-eGFP-STS-A3 and WR-eGFP-SKS-A4, the in vitro growth of recombinant VACV expressing core antigens fused with foreign antigens were reduced compared to wildtype VACV, affecting vaccine production efficiency. This problem could be due to the bulkiness of the foreign antigens chosen in our studies. Reducing the sizes of the fused antigen on a VACV core antigen may prevent any interference of VACV core packaging. Fusing foreign antigens to the carboxyl-terminus of the chosen core protein or choosing other core proteins could also be explored. Likewise, the use of other virion proteins as antigen carriers in VACV should be investigated to identify a more immunogenic substitute. The possibility of tagging multiple virion antigens with foreign antigens in a single VACV recombinant should also be explored. Secondly, the effect of immunodomination may still limit the use of heat-inactivated VACV as a recombinant vaccine vector. This study demonstrates that the anti-VACV $CD8^+$ T cell response is reduced after heat inactivation, but the responses against inserted antigens may compete with each other. For instance, the gB-498-specific $CD8^+$ T cell response was higher than those of

OVA-257 and D3E-128 after immunisation with heat-inactivated WR-eGFP-SKS-A4 or MVA- eGFP-SKS-A4. All three of these peptides are presented on H-2K^b. One possibility is that the gB-498-specific response has a strong immunodomination effect on responses specific to H-2K^b-binding peptides, compared to peptides that are presented on other MHC-I molecules (H-2D^b in C57BL/6 mice). MHC-I-specific immundomination has been detected during IAV infection in mice (Jenkins et al., 2006). To test whether immunodomination play a role here, new VACV recombinants that contain individual epitopes tagged onto the A4 protein can be generated and their ability to elicit CD8$^+$ T cell responses against the inserted peptides can be compared to WR-eGFP-SKS-A4 after heat inactivation.

Thirdly, whether heat-inactivated VACV can be used as a vaccine to induce protective immunity against heterologous pathogens needs to be investigated. We did not detect any significant enhancement of CD8$^+$ T cell responses against HSV gB-498 in mice primed with heat-inactivated WR-eGFP-STS-A3 compared to non-immunised mice after HSV-1 challenge. Similarly, immunisation with heat-inactivated WR-eGFP-STS-A3 did not reduce virus load in the HSV-1-infected ears from mice that were challenged with HSV-1 seven weeks after immunisation, compared to mice that were immunised with live wildtype WR (data not shown). More positively, priming mice with heat-inactivated WR-eGFP-STS-A3 and boosting with live WR-eGFP-STS-A3 elicited a better recall CD8$^+$ T cell responses against foreign antigens than a homologous prime-boost regime using only live WR-eGFP-STS-A3. Perhaps a higher anti-VACV antibody response might be induced in mice primed with live VACV than those immunised with the heat-inactivated virus, and this might result in a greater neutralisation of the booster vaccine. An advantage of our prime-boost strategy is that only one single vaccine is needed, overcoming the requirement for the use of different types of vaccine vectors as in a heterologous prime-boost strategy (Ramshaw and Ramsay, 2000; Woodland, 2004). Whether our prime-boost strategy could induce protective immunity against heterologous pathogen challenges, such as HSV-1 infection, should be examined. Fourthly, heat-inactivated MVA-eGFP-SKS-A4 seemed to prime a reduced level of CD8$^+$ T cells responses against the inserted antigenic peptides compared to WR-eGFP-SKS-A4. One possibility is that a

decreased amount of recombinant A4 was incorporated into the MVA virion particles compared to WR. The use of other virion antigens for MVA should be examined. In summary, heat-inactivated recombinant VACV is a new vaccine strategy that is worthy of further exploration.

Chapter 7 Concluding discussion

$CD8^+$ T cells play an important role in controlling infections caused by intracellular pathogens as well as tumours. Induction of protective $CD8^+$ T cell immunity with recombinant vaccines is the subject of ongoing research. In order to optimise the immunogenicity of a targeted antigen from a given recombinant vaccine vector, it is necessary to identify the priming pathway involved. Understanding the general rules determining priming pathway for a given vaccine vector would provide crucial information for designing better vaccines. This thesis focuses on the in vivo antigen presentation pathway for $CD8^+$ T cell priming by two strains of VACV, namely WR and MVA. Most of the basic research on VACV biology has used WR, although its clinical use is limited due to its virulence. MVA, in contrast, is now being used as a recombinant vaccine vector in clinical trials. Literature in the field suggests that the priming mechanisms for antigens expressed by these two strains of VACV differ. However, results shown in this thesis suggest that findings from WR can generally be applied to MVA. Importantly, the characteristics of the examined antigen dictate how an antigen is presented for $CD8^+$ T cell priming. Therefore, this thesis has implications for the design of VACV-based recombinant vaccines.

The first strategy used to dissect priming pathways of VACV in this thesis was to selective inhibit cross priming in vivo with the use of inhibitors, namely CpG and cytc (Chapter 3). However, treatment of mice with CpG and cytc, which have both been reported to selectively inactivate cross priming in vivo (Wilson et al., 2006; Lin et al., 2008), did not produce any meaningful insights regarding the priming mechanisms for individual native VACV antigens during VACV immunisation. Instead, the outcome of these two treatments was strongly linked to the replicative ability of the strain of VACV tested. For CpG treatment, we demonstrated that antigen dose was also an important factor dictating the ability of CpG to inhibit $CD8^+$ T cell priming. Furthermore, results with cytc implied that direct and cross presentation of viral antigens cannot be separated according to DC subsets. Although most published studies have focused on the cross priming ability of $CD8\alpha^+$ DCs (den Haan et al., 2000; Bedoui et al., 2009; Henri et al., 2010; Desch et al., 2011), $CD8\alpha^+$ DCs are likely to present peptides on MHC-I via direct presentation pathways as well (as discussed in Section 3.3). Elimination of these DCs by cytc may explain the inhibition observed of both pathways.

In the literature other mouse models have been suggested or already been used for dissecting antigen presentation pathways in vivo. Firstly, it has been suggested that Batf3-deficient mice, which lack both $CD8\alpha^+$ and $CD103^+$ DCs (Hildner et al., 2008), could be used as models to dissect antigen presentation (Yewdell, 2010; Hickman et al., 2011a). This is mainly based on the premise that these two types of DCs are the main DC subsets responsible for cross priming $CD8^+$ T cells. As discussed above, because of a potential role for these $CD8\alpha^+$ DCs in direct priming, it is highly questionable whether these mutant mice would constitute a reliable model for antigen presentation, especially for viruses that do not replicate in vivo. A further limitation on the use of this model for studying presentation of viral antigens is that it has been shown that development of functional $CD8\alpha^+$ DCs can be restored in these mice after infection with various pathogens (Tussiwand et al., 2012). This caveat would further complicate any meaningful analysis of $CD8^+$ T cell priming pathways using this model.

Secondly, two reports have shown that MyD88-deficient mice have a decreased ability to induce $CD8^+$ T cells responses against antigen donor cells infected with Semliki Forest virus and a soluble HSP fusion antigen (Palliser et al., 2004; Chen et al., 2005). MyD88 is the main adaptor signalling molecules for IL-1 receptors and TLRs, such as the TLR-9 mentioned in Section 3.1 (reviewed in Akira and Takeda, 2004). In DCs, MyD88 acts as the main switch for their maturation state after TLR ligation (Häcker et al., 2000; Schnare et al., 2000). However, MyD88-deficient DCs co-cultured with antigen-donor cells infected with Semliki Forest virus have a similar maturation status as wildtype DCs (Chen et al., 2005). In addition, MyD88-deficient DCs co-cultured with a HSP fusion antigen fused to a antigenic peptide had a reduced ability to re-stimulate a pre-activated peptide-specific $CD8^+$ T cell clone (which could simply be stimulated with a synthetic cognate peptide; Palliser et al., 2004). These results suggested that the lack of antigen processing and cross presentation of the captured antigens was responsible for the reduced $CD8^+$ T cell priming in the MyD88-deficient mice in the cross priming models examined. One study has found that MyD88 in DCs is important for recruiting TAP to the endosomes, a proposed compartment for MHC-I peptide loading in the phagosome-to-cytosol cross presentation pathway (Section 1.7.2.1;

Figure 1-3), as well as the subsequent MHC-I presentation of captured antigens in vitro in the presence of the TLR-4 ligand lipopolysaccharide (Burgdorf et al., 2008). However, results generated with this mutant mouse strain should be analysed with caution, especially during in vivo immunisation/infection models. This is because MyD88 is an important signalling molecule and it not only affects DCs, but also other components of the immune system. Importantly, expression of MyD88 in $CD8^+$ T cells is essential for their survival and expansion during VACV immunisation (Quigley et al., 2009; Zhao et al., 2009). In addition, it has been shown that DCs isolated from MyD88-deficient mice immunised with WR-TK-OVA could stimulate the proliferation of naive wildtype OT-I cells in vitro (Zhao et al., 2009). This suggested that the priming ability of MyD88-deficient DCs is not affected in the VACV immunisation model. Therefore, this model is not useful for dissecting presentation pathway of VACV antigens.

Thirdly, experiments with mice deficient for the Clec9A receptor have shown that this receptor is required for cross presentation of antigens associated with dead cells in vivo for $CD8^+$ T cell priming (Sancho et al., 2009; Zelenay et al., 2012). Although Clec9A is not involved in the uptake of dead cell materials, this receptor helps the transfer of captured dead-cell-associated antigens into endosomes that favour cross presentation of antigens (Sancho et al., 2009; Zelenay et al., 2012). Recently, it has been shown a reduced $CD8^+$ T cell response was induced in Clec9A-deficient mice immunised with VACV-infected cells, compared to wildtype mice (Iborra et al., 2012). Interestingly, the same study found that the anti-VACV $CD8^+$ T cell response induced by dermal VACV immunisation in these mutant mice was lower. More importantly, a VACV-strain-specific effect was observed, in which responses induced by MVA seemed to be affected to a greater extent than WR (Iborra et al., 2012). The authors suggested a role for cross priming of $CD8^+$ T cells during VACV immunisation (Iborra et al., 2012). These findings do not necessarily contradict our findings with the minigene constructs, which are purposely designed for enhancing direct presentation efficiency. It should be noted that the study by Iborra et al. (2012) demonstrated that direct presentation of VACV antigens was not affected in the Clec9A-deficient DCs in vitro. However, it remains unclear whether this is also true in vivo. This should be demonstrated empirically before a firm conclusion on the role of cross priming of

anti-VACV $CD8^+$ T cells can be drawn. VACV recombinants expressing the directly-presented OVA minigene could be used for this purpose (as in Chapter 3). Overall, in vivo presentation pathways for $CD8^+$ T cell priming cannot be easily separated based on the mouse models that eliminate cross presentation as discussed above.

Another strategy to dissect the presentation pathways for individual native VACV peptides was to use VACV recombinants to compensate for the antigen presentation deficiency in the mouse model examined. As mentioned in Section 3.1, Xu et al. (2010) generated MHC-I-deficient→wildtype bone marrow chimera, and then immunised these chimeric mice with a WR recombinant expressing H-$2K^b$, compensating for the loss of direct presentation ability of the infected APCs in vivo. From this experiment, it was demonstrated that the $CD8^+$ T cell response specific to B8-20 is induced by direct presentation by infected bone marrow-derived APCs (Xu et al., 2010). We have used a similar strategy to dissect how a large panel VACV peptides are presented for $CD8^+$ T cell priming in vivo. Instead of targeting peptides restricted to one MHC-I allele as in Xu et al. (2010), we decided to target both H-$2D^b$- and H-$2K^b$-presented VACV peptides by using TAP-deficient mice (Theodoratos et al., 2010). TAP-1→wildtype bone marrow chimera were generated. In these chimera, all DCs would be TAP-1-deficient and, therefore, be unable to directly present most peptides (excluding peptides that can be presented via a TAP-independent pathway). In addition, the proteasome-to-cytosol cross presentation pathway, which requires TAP (Ackerman et al., 2003; Guermonprez et al., 2003; Burgdorf et al., 2008), would be non-functional. If these mice were immunised with a TAP-1-expressing VACV, the direct presentation pathway would be rescued within infected cells. However, uninfected DCs within the immunised mice would still fail to cross present antigens. Using this model, we found that $CD8^+$ T cell responses against most peptides were significantly reduced in TAP-1→wildtype chimera immunised with a control WR compared with wildtype→wildtype chimera. When TAP-1→wildtype mice were immunised with WR expressing TAP-1, $CD8^+$ T cell responses against C4-125, A47-138, K3-6, and B8-20 were improved compared to the same chimera immunised with control WR (data not shown). These results suggested that these peptides are directly presented from infected bone-marrow derived APCs.

However, we later found that DCs originating from the wildtype recipients were not completely eliminated from the TAP-1→wildtype chimera, probably because of the irradiation dose used. This complicated the analysis of the results from the experiments and they were not included here (data not shown).

Nevertheless, this thesis demonstrates that $CD8^+$ T cells specific to antigens expressed from both strains of VACV, WR and MVA, can be effectively primed by direct presentation. Experiments with the directly-presented minigenes in Chapter 4 showed that these constructs expressed from WR and MVA are at least as potent as full-length antigens at inducing $CD8^+$ T cell responses after i.d. immunisation. These data suggest effective direct priming for antigens expressed from both VACV strains. This argument is supported by published studies with intravital microscopy, which showed direct interactions of naive $CD8^+$ T cells with DCs infected with WR or MVA (Norbury et al., 2002; Hickman et al., 2008; Kastenmüller et al., 2013). In addition, a recombinant MVA expressing CPXV12 and CPXV203, which suppress peptide translocation via TAP and MHC-I export to the cell surface respectively (Byun et al., 2007; Alzhanova et al., 2009; Byun et al., 2009), has been generated in our laboratory recently (Lin, 2013). Results with this virus broadly suggest a role for direct $CD8^+$ T cell priming during MVA immunisation in mice (Lin, 2013). Therefore, it is reasonable to conclude that the priming mechanism of antigens expressed from WR and MVA is indeed similar, being direct presentation.

Furthermore, our minigene experiments described above also argue against the suggestion that cross priming is the main pathway for $CD8^+$ T cell priming during i.d. immunisation (Shen et al., 2002), irrespective of the strain of VACV used. Infection of DCs in the draining LNs by WR and MVA occurs as early as two to four hours after local subcutaneous immunisation (Norbury et al., 2002; Hickman et al., 2008; Kastenmüller et al., 2013). Similarly, infectious WR virus can be isolated from draining LNs six hours after i.d. immunisation (Lin et al., 2013). These studies suggest that DCs can be directly infected with injected virus very early after a local immunisation, before the initial round of virus replication in vivo. Our finding that the non-replicating TMP/UV-treated WR-TK-SIIN induced strong responses against the directly-presented OVA-257 minigene (Section 3.2.6)

further indicates that direct priming of anti-VACV $CD8^+$ T cell responses does not require virus replication at the infection site during i.d. immunisation.

After local immunisation, VACV is likely to have direct access to draining LNs, especially when a high level of infectious virus is used, such as the normal doses used in this thesis. Within the draining LNs, mainly macrophages are infected but they do not support $CD8^+$ T cell priming (Norbury et al., 2002; Hickman et al., 2008; Hickman et al., 2011b). At the same time, some DCs are also infected which allow direct $CD8^+$ T cell priming. Alternatively, infected DCs from the infection site may migrate into the draining LNs for $CD8^+$ T cell priming. This phenomenon has been suggested after i.n. immunisation with VACV, where migratory $CD103^+$ DCs originating in the lungs from mice immunised with WR-NP-S-GFP supported the proliferation of naive OT-I $CD8^+$ T cell in vitro (Beauchamp et al., 2010). Whether this would occur during i.d. immunisation has yet to be tested. As discussed in Section 1.9.3, the DC subsets involved in $CD8^+$ T cell priming during VACV immunisation have been investigated in several studies (Belz et al., 2004; He et al., 2006; Abadie et al., 2009; Beauchamp et al., 2010; Liard et al., 2012). However, their findings were not consistent with each other. For instance, $CD8\alpha^+$ DCs were found to prime $CD8^+$ T cells when mice were infected with WR subcutaneously in the footpads or through the i.v. route (Belz et al., 2004; He et al., 2006). Contrary to these findings and as described above, Beauchamp et al. (2010) showed that migratory $CD103^+$ DCs, but not $CD8\alpha^+$ DCs, prime $CD8^+$ T cells after i.n. immunisation with WR. It is likely that the DC subset responsible for $CD8^+$ T cell priming is dependent on immunisation route.

Identification of the priming DCs during i.d. immunisation with VACV, which resembles human vaccination, remains crucial. As only when the physiological APCs are determined, can we start examining the relationship between in vitro presentation levels and in vivo priming capability of a given antigen expressed from VACV. To examine which DCs are involved in $CD8^+$ T cell priming after VACV immunisation via the i.d. route, an in vitro proliferation assay could be used. This involves isolation of individual subsets of DCs from the draining LNs from mice immunised i.d. with recombinant WR or MVA expressing the OVA-257 minigene, followed by co-culturing with CFSE-labelled naive OT-I cells in

vitro. The ability of each DC subset to sustain the proliferation of the naive T cells is assessed. This method has been commonly used to identify the priming DC subsets in various infection models (Belz et al., 2004; Belz et al., 2005; He et al., 2006; Bedoui et al., 2009; Beauchamp et al., 2010). One caveat of this assay is that the TCR transgenic $CD8^+$ T cells used, such as OT-I cells, may have a relatively lower activation threshold to cognate peptide stimulation compared to the wildtype $CD8^+$ T cell population (Zehn et al., 2009). In addition, naive purified $CD8^+$ OT-I cells can be activated simply by pulsing with synthetic OVA-257 peptide only (data not shown). Therefore, the in vitro proliferation assay with TCR transgenic $CD8^+$ T cells probably only reflects the level of MHC-I presentation on the DCs isolated, but not the genuine $CD8^+$ T cell priming that requires additional signals from antigen-presenting DCs, such as co-stimulatory signalling.

Another poxvirus genus that has clinical relevance in the context of vaccines is the avipoxvirus, including fowlpox virus and canarypox virus. Similar to MVA, these viruses only replicate in avian cells, but can infect mammalian cells abortively, allowing transgene expression (Taylor et al., 1988; Tartaglia et al., 1992; Somogyi et al., 1993; Pacchioni et al., 2010). These viruses are also well tolerated in immunodeficient mice (Leong et al., 1994). Therefore, research has been done to examine the usefulness of these viruses as recombinant vaccine vectors in clinical trials (Jäger et al., 2006; Kaufman et al., 2008; Rerks-Ngarm et al., 2009). It has been shown that $CD8^+$ T cell epitopes from the tumor-associated antigen, gp100, expressed as minigenes from canarypox virus are able to induce $CD8^+$ T cell responses in mice (Tine et al., 2005). In an independent study, the immunogenicity of recombinant fowlpox viruses expressing a gp100 $CD8^+$ T cell epitope as an ER-targeted minigene or as a full-length antigen were examined in human patients (Rosenberg et al., 2003). In both constructs, the encoded epitope was modified to enhance the binding affinity to the restricting MHC-I (Rosenberg et al., 2003). The study found that after two rounds of immunisation, fowlpox virus expressing the ER-targeted minigene induced an epitope-specific $CD8^+$ T cell response in more human patients than fowlpox virus expressing the full-length antigen (Rosenberg et al., 2003). Overall these reports suggest that

minigenes expressed from non-replicating poxviruses can prime $CD8^+$ T cell responses effectively, in accordance with the experiments with MVA shown here.

While antigen presentation pathways seem unaffected by poxvirus strain, this thesis suggests a major role for the characteristics of a given antigen in dictating how the antigen is presented. Firstly, only some, not all, native VACV antigens can be cross presented from VACV-infected cells or viral particles in vivo (Chapters 3 and 6). For all recombinant antigens examined, only full-length stable constructs can be cross presented (Chapters 4). For instance, it has been found that the PB1F2 antigen is unstable (Chen et al., 2001a), and this may explain why no PB1F2-62-specific $CD8^+$ T cell response was induced by cells infected with WR or MVA expressing full-length PB1F2. Moreover, all minigenes examined in this thesis fail to be cross presented in vivo from infected cells. Interestingly, Lev et al. (2008) found that the PA-224 minigene expressed in VACV-infected cells can induce $CD8^+$ T cell responses via cross priming. This is because the minimal peptide is stabilised within infected cells (Lev et al., 2008), allowing it to survive long enough for cross presentation. This discovery is an exception to the findings shown in this thesis and published work (Norbury et al., 2004; Basta et al., 2005; Lev et al., 2008), representing only one out of seven minigenes expressed from VACV that have been examined to our knowledge.

Besides suitability for cross priming, various characteristics of a given antigen can also influence the immunogenicity of the directly-presented constructs. For instance, results in Chapter 5 show that the immunodominant NP-366 from IAV was much less immunogenic when expressed from WR as constructs that facilitate direct presentation, contradicting the general rule reported for other antigens in Chapter 4, as well as the vast number of published studies (Irvine et al., 1995; Restifo et al., 1995; Tobery and Siliciano, 1997; Fu et al., 1998; Tobery and Siliciano, 1999; Norbury et al., 2004). Similar to our results with the PR8NP-366 peptide from WR-UbR-NP-S-GFP, a recent report showed that expressing the LCMV NP antigen as a rapidly-degraded construct from WR was less immunogenic and resulted in a reduction of $CD8^+$ T cell responses against two $CD8^+$ T cell epitopes available on the antigen in C57Bl/6 mice (Schliehe et al., 2012). However, a minigene version of one of the two $CD8^+$ T cell epitopes from

the LCMV NP antigen tested has been found to be immunogenic in vivo when expressed from WR (Probst et al., 2003). Therefore, the mechanism involved might be different from that of IAV NP, which was poorly immunogenic when expressed as a minigene or a rapidly-degraded construct. Based on the results and studies discussed above, it is reasonable to conclude that the nature of a given antigen, but not the strain of VACV, governs the priming pathway used. Therefore, although there may be general rules that are applicable to many epitopes, each of them must be examined individually to identify the optimal constructs.

It should be noted that for most of the recombinant viruses used in this thesis, only one single final recombinant was generated (except for MVA-delIII-SIIN-delTK used in Section 4.2.6). Multiple rounds of plaque purification are involved in generating a VACV recombinant and it is possible that a virus with unfavourable random mutations might be inadvertently selected. Without an independent biological replicate, this possible adverse event cannot be controlled. In this thesis, this problem was overcome by studying several recombinant antigens and focusing on the general conclusions. However, for effects observed with individual recombinants, such as the reduced overall immunogenicity of WR-delB8-miniB8 observed in Section 4.2.3, it would be unwise to conclude that the observed phenomenon was only due to the intended mutation. In these cases, a second independent recombinant virus is required. This important control is rarely considered in most studies in the field of vaccinology and immunology which use recombinant viruses. This practice contrasts greatly with that of virology, where the use of independent lineages of recombinant viruses or revertant viruses is the norm.

This thesis also has implications for designing VACV-based recombinant vaccines. Firstly, the insertion site, and possibly function of VACV TK, modulates the relative immunogenicity of the minigenes compared with the full-length antigens, highlighting the importance of vector design. This explained the disparity between the high immunogenicity of minigenes expressed from the TK locus of MVA, as shown in Chapter 4, and the study by Gasteiger et al. (2007), which found that the OVA-257 minigene expressed from the delIII region was

less immunogenic than full-length OVA. Although the mechanism responsible has not been identified, results here suggest that insertion site should be chosen based on the immunogenicity of the inserted antigens, not because of historical precedent or a simple rationale to preserve all VACV genes.

Secondly, in this thesis as well as most published studies, the possibility that the functions of foreign antigens expressed from VACV could affect the immune responses induced was not examined. As minigenes are unlikely to retain any functional capacity, this is probably more relevant to full-length antigens than minigenes. It is known that antigens that were intended to be used as priming substrates for inducing immune responses can exert unforeseen functions. For instance, insertion of transgenes expressing HIV envelope (Env) protein and a group-specific-antigen-polymerase (Gag-Pol) fusion protein into MVA can result in an unstable recombinant virus (Wyatt et al., 2009). It has been suggested that expression of the HIV genes is detrimental to VACV replication, and so they pose a strong selective pressure for recombinant viruses that do not express the inserted genes (Wyatt et al., 2009). Similarly, expression of an parainfluenza virus 3 antigen from WR or MVA also significantly reduces virus replication in vitro (Wyatt et al., 1996). Expression of the HIV Env and Gag-Pol proteins from MVA enhances apoptosis of the infected cells (Zhang et al., 2007). How this would affect the induction of a immune response has not been examined. One might assume that cross priming might be the dominant pathway for inducing $CD8^+$ T cell responses against the inserted antigens if such a recombinant virus was used as DCs infected with the virus might die before allowing proper $CD8^+$ T cell priming. In addition, antigens within necrotic cells and apoptotic cells could be captured and processed by different cross presentation pathways (Albert et al., 1998; Sauter et al., 2000; Sancho et al., 2009). The nature of cellular death could influence the outcome of cross priming. Therefore, the influence of foreign antigens expressed from vaccine vectors on the biology of infected cells and, as a result, antigen presentation should not be overlooked.

Thirdly, there are also implications for the clinical translation of research from mice to human use. Because different epitopes are targeted to murine and human MHC-I, they may be processed and presented by different pathways even when

expressed from the same antigen. Transgenic mice expressing human MHC-I molecules, such as HDD mice (Pascolo et al., 1997), have been used as an in vivo model to investigate $CD8^+$ T cell responses against epitopes specific to human MHC-I during viral infection (Wentworth et al., 1996; Drexler et al., 2003; Kotturi et al., 2009; Tan et al., 2011). However, it should be noted that the details of antigen processing and presentation pathways in these mice will be different from that of human. Indeed, it has been shown that the specificity of proteasomal processing, TAP translocation, and peptide-loading with tapasin are slightly different between humans and mice (Neefjes et al., 1995; Sesma et al., 2003). Therefore, care should be taken when information obtained from mice are to be used for designing vaccines for use in humans. Furthermore, the results with IAV NP-366 peptide shown in Chapter 5 suggest that in vitro presentation level does not necessarily reflect $CD8^+$ T cell priming in vivo. As a result, although the use of human cells to assess in vitro presentation levels may be useful for dictating possible priming outcomes for some antigens, care should be taken when translating the in vitro results for clinical uses.

Fourthly, we have shown a potential use of heat-inactivated VACV as a carrier for recombinant antigens to induce $CD8^+$ T cell responses. This was done by creating VACV recombinants with foreign peptides of interest attached to virion antigens. Even after complete inactivation of these VACV recombinants, we have shown that $CD8^+$ T cell responses can be induced against the attached peptides in vivo (Chapter 6). The induction of responses against native VACV peptides was lowered in these inactivated viruses because most VACV antigens are not expressed by the inactivated virus in vivo and are not incorporated into VACV virions. This reduction of responses against vector could reduce the immunodomination of those directed towards the inserted antigen. A similar notion has been made previously in which minigene-expressing recombinant VACVs were treated with psoralen/UV (Fischer et al., 2007). This treatment selectively suppressed expression and MHC-I presentation of long VACV antigens (Fischer et al., 2007). Furthermore, the anti-VACV $CD8^+$ T cell response was reduced in mice immunised with psoralen/UV-treated WR-TK-OVA and resulted in an enhancement of the response against the OVA-257 minigene (Fischer et al., 2007). This study supports the idea that reduction of the anti-vector

response would be beneficial to the responses against the foreign antigens of interest.

To summarise, results in this thesis demonstrate a general principle that direct presentation is highly effective for inducing $CD8^+$ T cell responses against antigens expressed from VACV strains WR and MVA. However, as for most of biology, exceptions to this general rule exist. Such deviations need to be taken into account during the examination of individual antigens and design of recombinant vaccine vectors.

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Appendix

Viruses used in Chapter 3 are shown in Table A-1. The information shown is reproduced from Section 2.1.8.

Table A-1| Viruses used in Chapter 3

Virus	Description
WR	Wildtype WR
MVA	Wildtype MVA
WR-TK-SIIN	WR expressing a cytosolic OVA-257 minigene by the VACV p7.5 promoter from the TK locus
WR-TK-OVA	WR expressing full-length OVA by the VACV p7.5 promoter from the TK locus
WR-NP-S-GFP	WR expressing a recombinant NP-S-GFP antigen consisting of PR8NP, OVA-257 peptide and eGFP by the p7.5 promoter from the TK locus
MVA-TK-SIIN	MVA expressing a cytosolic OVA-257 minigene by the VACV p7.5 promoter from the TK locus

Table A-2 shows some information regarding individual native VACV antigens that contain $CD8^+$ T cell epitopes examined in this thesis. Differences in the putative promoter sequences for the corresponding VACV genes and the amino acid sequences of the antigens between WR and MVA are listed.

Table A-2| Conservations of VACV antigens expressed from WR and MVA

VACV peptide	Peptide sequence	Changes in the promoter and protein in MVA (relative to WR)	
		Promoter [1]	Protein [2]
A42-88	YAPVSPIVI	insA-18	del45-49
A8-70	IHYLFRCV	Nil	Nil
J3-289	SIFRFLNI	A-77G, A-73G	Nil
C4-125	LNFRFENV	The whole C4L gene is deleted from the MVA genome	
A3-191	YSPSNHHIL	G-44A, C-85T	Q51K
A47-171	YAHINALEYI	del-36	del29-33, K71E, del153-161, M236I
L2-53	VIYIFTVRL	Nil	T5A
A47-138	AAFEFINSL	Refer to A47-171	
B2-54	YSQVNKRYI	The MVA B2 antigen is truncated at position 32, therefore the B2-54 peptide is not expressed from MVA	
K3-6	YSLPNAGDVI	Nil	K22N, F36S
A23-297	IGMFNLTFI	Nil	P58H
A3-270	KSYNYMLL	Refer to A3-191	
A8-189	ITYRFYLI	Refer to A8-70	
B8-20	TSYKFESV	Nil	del95-102, del115-121, trnc241

1 Changes in the 85 bp upstream of the start codon (ATG) of the gene are shown. The number denoted the position upstream from the start codon. del: deletion; ins: insertion.

2 del: deletion; Trnc: truncation, the sequence after the amino acid position shown is not produced.

Viruses used in Chapter 4 are listed in Table A-3. The genetic modifications that were introduced into the viruses are shown.

Table A-3| Viruses used in Chapter 4

Virus	Genetic modification		
	Recombinant antigen expressed [1]	Insertion site	Gene knocked out
WR-Full-gB	Full-length HSV gB	TK	TK
WR-ESmini-gB	ER-targeted gB-498 minigene	TK	TK
WR-delTK	-	-	TK
MVA-Full-gB	Full-length HSV gB	TK	TK
MVA-ESmini-gB	ER-targeted gB-498 minigene	TK	TK
MVA-delTK	-	-	TK
WR-Full-PA	Full-length IAV PA	TK	TK
WR-ESmini-PA	ER-targeted PA-224 minigene	TK	TK
WR-Full-PB1	Full-length IAV PB1	TK	TK
WR-ESmini-PB1	ER-targeted PB1-703 minigene	TK	TK
WR-Full-NS2	Full-length IAV NS2	TK	TK
WR-ESmini-NS2	ER-targeted NS2-114 minigene	TK	TK
WR-Full-PR8NP	Full-length IAV PR8NP	TK	TK
WR-ESmini-PR8NP	ER-targeted PR8NP-366 minigene	TK	TK
WR-Full-PB1F2	Full-length IAV PB1F2	TK	TK
WR-ESmini-PB1F2	ER-targeted PB1F2-62 minigene	TK	TK
MVA-Full-PB1F2	Full-length IAV PB1F2	TK	TK
MVA-ESmini-PB1F2	ER-targeted PB1F2-62 minigene	TK	TK

Table A-3 (Continued)| Viruses used in Chapter 4

Virus	Genetic modification		
	Recombinant antigen expressed [1]	Insertion site	Gene knocked out
MVA-ESmini-PB1F2	ER-targeted PB1F2-62 minigene	TK	TK
WR	-	-	-
WR-delB8-miniB8	Cytosolic B8-20 minigene	B8R	B8R
WR-delB8	-	B8R	B8R
MVA	-	-	-
MVA-delB8-miniB8	Cytosolic B8-20 minigene	B8R	B8R
MVA-delB8		B8R	B8R
WR-TK-OVA	Full-length OVA	TK	TK
WR-TK-SIIN	Cytosolic OVA-257 minigene	TK	TK
MVA-delIII-OVA	Full-length OVA	delIII region	-
MVA-delIII-SIIN	Cytosolic OVA-257 minigene	delIII region	-
MVA-TK-OVA	Full-length OVA	TK	TK
MVA-TK-SIIN	Cytosolic OVA-257 minigene	TK	TK
MVA-A11/A12-OVA	Full-length OVA	A11R/A12L intergenic region	-
MVA-A11/A12-SIIN	Cytosolic OVA-257 minigene	A11R/A12L intergenic region	-
MVA-delIII-OVA-delTK	Full-length OVA	delIII region	TK
MVA-delIII-SIIN-delTK	Cytosolic OVA-257 minigene	delIII region	TK

1 It should be noted that for most of the recombinant viruses expressing transgenes from the TK locus, a *lacZ* gene under the control of the late VACV p11 promoter was also inserted into the TK locus, except for the recombinant viruses WR-Full-PA and WR-Full-NS2.

Table A-4 lists all the viruses used in Chapter 5. The information is reproduced from Section 2.1.8. All the WR recombinants used express a *lacZ* gene under the control of the late VACV p11 promoter from the TK locus.

Table A-4| Viruses used in Chapter 5

Virus	Recombinant antigen expressed
WR-Full-PR8NP	Full-length PR8NP
WR-ESmini-PR8NP	ER-targeted PR8NP-366 minigene [1]
WR-mini-PR8NP	Cytosolic PR8NP-366 minigene
WR-Full-NT60NP	Full-length NT60NP
WR-ESmini-NT60NP	ER-targeted NT60NP-366 minigene [2]
WR-NP-S-GFP	NP-S-GFP antigen
WR-UbR-NP-S-GFP	NP-S-GFP antigen tagged with an ubiquitin protein and an arginine residue at the amino-terminus
WR-cytc-PR8NP	A recombinant antigen consisting of the amino acid residues of 13-498 of PR8NP (PR8NP13-498)
WR-ES-PR8NP	ER-targeted PR8NP13-498 [3]
WR-delTK	-
IAV strain PR8	-

1 The ER-targeted signal peptide from the adenovirus E3/19K protein was used.
2 The ER-targeted signal peptide of the hemagglutinin from IAV strain PR8 was used.
3 The ER-targeted signal peptide of the hemagglutinin from IAV strain A/WSN/33 was used.

The viruses used in Chapter 6 are listed in TableA-5. The information is reproduced from Section 2.1.8.

Table A-5| Viruses used in Chapter 6

Virus	Recombinant antigen expressed
WR	-
MVA	-
WR-eGFP-STS-A3	WR, expressing a recombinant GFP-STS antigen, attached to the amino-terminus of A3 protein
WR-eGFP-SKS-A4	WR, expressing a recombinant GFP-SKS antigen, attached to the amino-terminus of A4 protein
MVA-eGFP-SKS-A4	MVA, expressing a recombinant GFP-SKS antigen, attached to the amino-terminus of A4 protein

Philippians 3:13
"Forgetting those things which are behind, and reaching forth unto those things which are before"